BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : A61K 49/02 — A1

(11) International Publication Number: WO 90/10463

(43) International Publication Date: 20 September 1990 (20.09.90)

(21) International Application Number: PCT/US90/01399

(22) International Filing Date: 14 March 1990 (14.03.90)

(30) Priority data:
324,285 — 14 March 1989 (14.03.89) — US
364,687 — 9 June 1989 (09.06.89) — US

(71) Applicant: NEORX CORPORATION [US/US]; 410 West Harrison, Seattle, WA 98119 (US).

(72) Inventors: MORGAN, A., Charles, Jr. ; 803 Driftwood Place, Edmonds, WA 98020 (US). ANDERSON, David, C. ; 2353 West Halladay, Seattle, WA 98199 (US).

(74) Agents: ZELANO, Anthony, J. et al.; Millen, White & Zelano, Arlington Courthouse Plaza I, Suite 1201, 2200 Clarendon Boulevard, Arlington, VA 22201 (US).

(81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: IMAGING TISSUE SITES OF INFLAMMATION

(57) Abstract

The present invention involves methods of enhancing the amount of label accumulating at tissue sites of inflammation. Methods of the present invention take advantage of the up-regulation of surface antigenic markers on leukocytes upon activation thereof. Imaging applications of such enhancement are described.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MR	Mauritania
BE	Belgium	GA	Gabon	MW	Malawi
BF	Burkina Fasso	GB	United Kingdom	NL	Netherlands
BG	Bulgaria	HU	Hungary	NO	Norway
BJ	Benin	IT	Italy	RO	Romania
BR	Brazil	JP	Japan	SD	Sudan
CA	Canada	KP	Democratic People's Republic of Korea	SE	Sweden
CF	Central African Republic			SN	Senegal
CG	Congo	KR	Republic of Korea	SU	Soviet Union
CH	Switzerland	LI	Liechtenstein	TD	Chad
CM	Cameroon	LK	Sri Lanka	TG	Togo
DE	Germany, Federal Republic of	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		

IMAGING TISSUE SITES OF INFLAMMATION

Technical Field of the Invention

The present invention relates to the field of diagnostic imaging. More specifically, the invention involves improved imaging of tissue sites of inflammation. Improved diagnostic images result from an increase in the number of labeled leukocytes in the area of the inflammation or from improved selectivity of antibodies or peptides for activated leukocytes in sites of inflammation versus non-activated leukocytes in the circulation.

Background Art

Inflammation occurs as a result of infection with a microorganism, tissue injury, or, as has been recently recognized, in non-apparent tissue injury associated with transient ischemia. A major application of imaging agents targeted to inflammation has been the imaging of abscesses due to regional replication of microorganisms.

Two general methodologies have been developed for imaging abscesses caused by replication of infectious organisms like bacteria or fungi. The first relies on detection of antigens expressed by the bacteria or fungi. In this case, antigen expressed by the microorganism itself is the target for imaging by antibody. The second method makes use of the fact that growth of infectious organisms will cause inflammation. The inflammation process then can be used as a target for imaging.

The most utilized method for imaging inflammation is one in which polymorphonuclear leukocytes (PMNs) or unfractionated leukocytes are isolated from a patient and labeled with radionuclides (for instance, with ^{111}In). The labeled autologous leukocytes are then reinjected into the donor. A certain percentage of the labeled PMNs will accumulate at the sites of abscess formation or

inflammation. However, many drawbacks have been experienced using this methodology. One such difficulty relates to the labeling methodologies and their effect on leukocyte trafficking. Oxidative processes used in the labeling procedure may cause the PMNs to be more effectively removed by the reticuloendothelial system (RES) which has as its normal function the recognition, removal and destruction of effete cells in the body. Thus, in scans obtained by such labeling methodologies a substantial accumulation of labeled cells in the liver, spleen, and other RES sites is commonly observed. This RES accumulation detracts from a diagnostician's ability to detect inflammatory lesions within RES organs. Such accumulation also reduces the number of labeled cells that can accumulate at the site of the abscess (bioavailability), and thus decreases the sensitivity of inflammation detection in organs outside of the RES.

One abscess imaging methodology which has been suggested as an improvement involves a non-oxidative method of labeling the cells, making use of radiolabeled antibodies which bind to surface antigens of PMNs. Antibodies are labeled with a radioisotope suitable for imaging, and the antibody is then incubated with isolated PMNs or leukocytes prior to reinjection of the autologous cells into the donor. The method is an improvement because of its simplicity, but might also improve the number of leukocytes that can localize to abscesses because of reduced labeled cell accumulation in the RES system. Even with this improvement, only a small percentage of the labeled leukocytes will actually localize to the tissue sites of inflammation. Thus, there is a need for improved methods for enhancing accumulation of labeled leukocytes, and more specifically PMNs, into abscesses and sites of tissue inflammation.

Other potential methods for imaging abscesses or sites of inflammation use passively administered anti-

bodies to localize to sites of inflammation. Such uses have been postulated for monoclonal antibodies directed to activation antigens expressed on monocytes which have matured into macrophages.

Summary of the Invention

The present invention serves to improve upon diagnostic images described in the prior art by enhancing the amount of label associated with leukocytes accumulating at inflamed tissue sites, such as inflammatory lesions or abscesses. This enhancement of label at sites of tissue inflammation is achieved by infusing labeled recognition agents capable of interacting at the site of inflammation with leukocytes which have been activated during the inflammatory process. The labeled recognition agents exhibit an ability to traverse the vascular system and enter the tissue site to be imaged.

Enhanced label accumulation at the target tissue site may be accomplished in accordance with the present invention by paving the way for label through the bloodstream and peripheral tissue. That is, a non-labeled recognition agent is infused first to bind to these peripheral sites to permit more rapid and complete accumulation of later administered, labeled recognition agent at sites of inflammation.

The present invention encompasses imaging methods which employ as recognition agents monoclonal antibodies and peptides capable of interacting with receptors that have augmented expression on activated leukocytes. Monoclonal antibodies useful in the present invention are directed against epitopes of cell surface antigens which are up-regulated upon leukocyte activation. Thus, the monoclonal antibodies can interact with activated leukocytes located at sites of tissue inflammation. Monoclonal antibodies which are directed against activated leukocytes and do not exhibit substantial binding to non-

activated leukocytes or exhibit a greater than 10-fold preference for activated leukocytes are especially useful recognition agents of the present invention. Imaging methods which utilize photoaffinity labels are also contemplated.

Also, imaging methods which feature ex vivo activation of autologous leukocytes and incubation of these activated leukocytes with labeled recognition agent are contemplated by the present invention. Infusion of both the leukocytes and labeled recognition agent into a patient following incubation of the same serves to enhance the accumulation of label at tissue sites of inflammation.

Detailed Description of the Invention

The primary function of polymorphonuclear leukocytes (PMNs) is the protection of a host against invasion by pathogenic organisms, such as bacteria or fungi. Other leukocytes, such as the monocyte, are additionally involved in this protective mechanism. Monocytes transformed into mononuclear phagocytes at the tissue site also participate in the protective process.

When pathogenic organisms become established in a host and begin proliferating, the infected host typically undergoes an inflammatory response. This inflammatory response is characterized by dilation of the blood vessels in the vicinity of the microorganism proliferation, increased vascular permeability in that area, and the movement of leukocytes, such as monocytes and PMNs, from the bloodstream into the infected tissue site. The increase in the volume of blood flowing past or through the area of infection; the increased ease of cellular passage through the blood vessel to the infected tissue; and the migration of phagocytic cells from the bloodstream to the infected area represent the host's response to the pathogenic organism's invasion.

PMNs and monocytes/mononuclear phagocytes which accumulate at a tissue site infected with such pathogenic organisms will provide an immune response through phagocytosis. That is, mononuclear phagocytes and PMNs will ingest the pathogenic cells and kill the ingested pathogens internally. Consequently, the greater the number of PMNs and mononuclear phagocytes that migrate to the infected area, the higher the rate of phagocytosis.

This migration of PMNs and mononuclear phagocytes to tissue sites that are inflamed also has ramifications for diagnostic imaging. By associating an imagable label with these mononuclear phagocytes and PMNs, an image of the infected area may be obtained. As with phagocytosis, the greater the number of PMNs and mononuclear phagocytes that migrate to the infected areas, the better the image.

The first aspect of the present invention involves a method of imaging tissue sites of inflammation comprising:

(1) labeling a recognition agent, wherein said agent is capable of interacting selectively with activated leukocytes accumulated at said tissue site;

(2) infusing labeled recognition agent into a patient; and

(3) imaging said tissue sites,

whereby medical conditions involving tissue damage mediated by inflammation may be detected, evaluated and monitored.

By imaging there is contemplated conventional diagnostic in vivo imaging. Briefly, a substance which is capable of detection within a patient, i.e., a labeled substance such as a radionuclide labeled antibody, is administered to a patient in an amount sufficient to deliver an adequate supply of labeled substance to the target tissue so as to permit an image to be generated. The radionuclide provides the imaging input, while the

coupled (labeled) substance provides the targeting capability of the radiolabeled unit.

A tissue site of inflammation is one which exhibits tissue damage mediated by inflammation. Thus, a tissue site of inflammation may be one where damage to tissue prompts an inflammatory response. Alternatively, the damage to a tissue site may be exacerbated or be generated by an inflammatory response itself. In the first instance, a patient initially suffers tissue damage and then his immune system mounts an inflammatory response to that damage. In the second scenario, the inflammatory response induced by transient ischemia, for example, causes or exacerbates the tissue damage.

Exemplary of tissue damage mediated by inflammation are infectious agent multiplication and tissue abscesses. When infectious agents are involved, tissue damage may result from actions of the proliferating invader cells (the first situation described above) or from the inflammatory response (the second situation described above). By infectious agent, there is contemplated any pathogenic organism. Exemplary of such organisms are bacteria, viruses and fungi. Another example of tissue damage mediated by inflammation is the damage suffered by ischemic heart muscle brought about by myocardial infarction, since transient decreases in blood flow to tissue sites result in minimal tissue damage which, however, is sufficient to induce leukocyte activation and subsequent inflammation.

The labeling of the present invention may be accomplished by covalently or noncovalently linking a moiety which generates an input for an imaging technique with a recognition agent. The label-recognition agent conjugate will be administered to the patient. Exemplary of labels useful in the present invention are radionuclides. This labeling may be done by conventional techniques. For

example, Alvarez et al. suggest methodologies for such labeling in U.S. Patent No. 4,741,900.

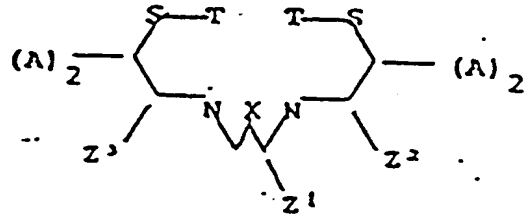
Radionuclides useful within the present invention include gamma-emitters, positron-emitters and fluorescence emitters. Exemplary radionuclides are well-known in the art and include ^{111}In, ^{198}Au ^{113}Ag, ^{111}Ag, ^{123}I, ^{125}I, ^{130}I, ^{131}I, ^{133}I, ^{135}I, ^{47}Sc, ^{72}As, ^{72}Se, ^{90}Y, ^{88}Y, ^{97}Ru, ^{100}Pd, ^{109}Pd, ^{105}Rh, ^{128}Ba, ^{197}Hg, ^{203}Pb, ^{212}Pb, ^{67}Ga, ^{68}Ga, ^{64}Cu, ^{67}Cu, ^{97}Ru, ^{75}Br, ^{76}Br, ^{77}Br, ^{99m}Tc, ^{11}C, ^{13}N, ^{15}O and ^{18}F.

The present invention also contemplates radionuclide labeling of a recognition agent via a chelating compound. A chelating compound is a moiety capable of complexing with a radionuclide. Exemplary chelating compounds are those described in published European Patent Applications numbers 0188256, 0289187 and 0203764.

Exemplary chelating compounds include compounds containing various combinations of nitrogen and sulfur atoms which act as the donor atoms for binding a metal or metal oxide. European Patent Application 0188256 discloses representative chelating compounds and their synthesis. A chelating compound within the present invention may be a compound having four to six nitrogen and sulfur donor atoms. One example of a chelating compound containing two nitrogens and two sulfurs is referred to herein as "N_2S_2". Other chelating compounds included within the invention have different numbers of nitrogen and sulfur atoms. Examples of these chelating compounds are identified in like manner herein as "N_3S", "N_2S_3", "N_2S_4" and "N_3S_3". Each of these representative chelating compounds is described in more detail below. In addition, the following U.S. Patent Applications are hereby incorporated in their entirety by reference: U.S.S.N. 065,017 (filed June 19, 1987) "Metal Radionuclide Labeled Proteins For Diagnosis And Therapy"; U.S.S.N. 172,004 (filed March 23, 1988) "Metal Radionuclide-Labeled Proteins And Glycoproteins For Diagnosis

And Therapy"; U.S.S.N. 201,134 (filed May 31, 1988) "Metal Radionuclide Chelating Compounds For Improved Chelation Kinetics"; and U.S.S.N. 157,284 (filed February 17, 1988) "Anchimeric Radiometal Chelating Compounds."

The N_2S_2 metal chelating compounds may be dithio, diamino, diamidocarboxylic acids; amino/thio/amido combinations or derivatives thereof, e.g., a N,N'-bis-mercaptoacetyl, diamino carboxylic acid; esters capable of forming an amide bond in an aqueous medium; and intermediates of these esters. An example of N_2S_2 metal chelating compounds has the following formula:



wherein:

one of Z^1, Z^2, Z^3 or Z^4 is RCW-$(HNV)_nY$, and the others are =O or H_2;

R is a divalent organic radical of at least 1 carbon atom and typically not more than 10, usually not more than 6 carbon atoms, usually from 1 to 3 carbon atoms, having from 0 to 2 heteroatoms which are chalcogen (O, S) or nitrogen, and is aliphatic, alicyclic, aromatic or heterocyclic (preferably aliphatic having from 0 to 2, usually 0 to 1 site of aliphatic unsaturation, e.g., ethylenic, and containing 1 to 2 carbon atoms);

W is oxygen or imino (=O or =NH), with the proviso that when Y is $-NH_2$ or $-NHNH_2$, the W bonded to the carbon atom bonded to Y is H_2;

V is RCW, where the two RCW groups may be the same or different, usually being of from 1 to 8, more usually of from 1 to 6 carbon atoms, preferably of from 2 to 3 carbon atoms;

n is 0 or 1;

T is an acyl or acylthio radical of from 2 to 10, usually 2-8 carbon atoms, either a hydrocarbyl acyl or substituted acylradical, usually aryl (e.g., phenyl) or alkyl (e.g., methyl); an organic sulfhydryl radical of from 1 to 10 carbon atoms, either substituted or unsubstituted hydrocarbyl; a heterocycle, particularly a chalcogen (O, S) heterocycle; an acylamidomethylene, where the acyl group is as defined above; hydrogen; sulfonato; an alkali metal ion; or the two T's may be taken together to define a polyvalent metal radionuclide, as the metal ion or metal ion oxide;

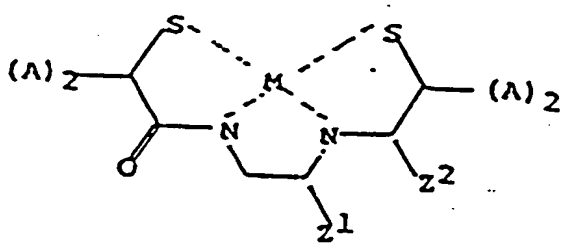
Substituents include nitro, cyano, halo, non-oxocarbonyl, carboxylic acid, amide and ester, and the like;

Y is a chemically reactive moiety capable of reacting with a recognition agent to bind the chelate thereto as is defined below;

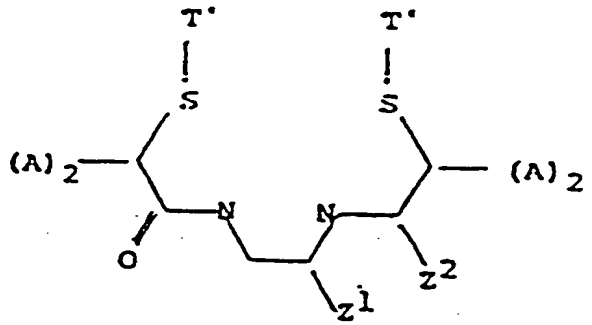
A's are the same or different and are hydrogen, carboxylate or lower alkyl of from 1 to 6 carbon atoms, usually of from 1 to 3 carbon atoms, particularly methyl, or hydrogen; and

X is a bond, methylene or CHZ^4.

A preferred group of N_2S_2 compounds will have one of the following formulae:



or

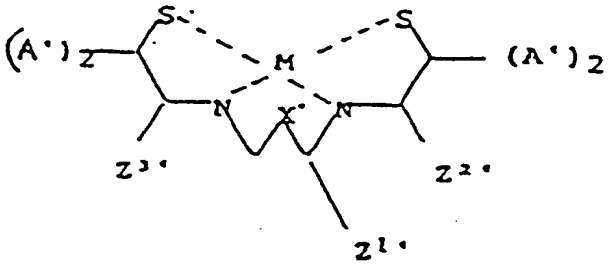


wherein all of the symbols are as defined previously except for M and T', and wherein:

M is a radionuclide capable of being chelated as the metal ion or metal ion oxide; and

T' is a sulfur protective group, which includes acyl, acylthio, hydrocarbylthio or substituted-hydrocarbylthio or heterocyclicthio, where the acyl and hydrocarbyl groups may be aliphatic, alicyclic, aromatic or combinations thereof and the acyl group further includes heterocyclic, wherein acyl is normally carboxyacyl; T' will generally be of from 2 to 10 carbon atoms, usually 2 to 8 carbon atoms when acyl, where substituents will include non-oxo-carbonyl, halo, particularly fluoro and chloro, cyano and nitro.

N_2S_2-type chelate compounds will for the most part have the following formula:



wherein:

one of $Z^{1'}$, $Z^{2'}$, $Z^{3'}$ or $Z^{4'}$ is R'CW' $(HNV')_{n'}Y'$, and the others are =O or H_2;

(A')'s are the same or different and are hydrogen, carboxylate or lower alkyl of from 1 to 6, usually 1 to 3 carbon atoms, particularly methyl, usually hydrogen;

n' is 0 or 1;

V' is R'CW', where the (R'CW)'s may be the same or different, usually being of from 1 to 8, more usually of from 1 to 6 carbon atoms, preferably of from 2 to 3 carbon atoms;

W' is oxygen or imino (=N or =O), with the proviso that when Y' is $-NH_2$ or $-NHNH_2$, the W' bonded to the carbon atom bonded to Y' is H_2;

M is a radionuclide capable of being chelated as the metal ion or metal ion oxide;

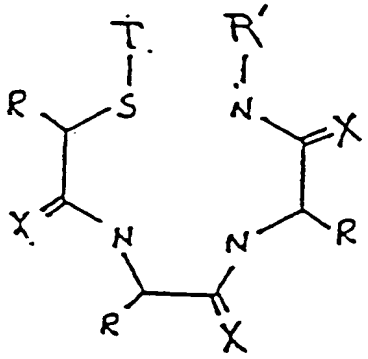
X' is a bond, methylene or CHZ^4;

R' is an aliphatic divalent radical of from 1 to 6, usually from 1 to 3 carbon atoms, having from 0 to 1 site of aliphatic unsaturation and 0 to 2 heteroatoms, usually straight chain and preferably methylene or polymethylene of from 2 to 3 carbon atoms; and

Y' is a chemically reactive moiety capable of reacting with a recognition agent to bind the chelate thereto as defined below.

The dashed lines in the formulae presented for the chelate compounds of the invention represent four coordinate covalent bonds between the metal radionuclide M and each of the two sulfur and the two nitrogen atoms shown in the formulae. Thus, the metal radionuclide is bound through relatively stable bonds in the chelate compounds of the invention.

N_3S metal chelating compounds will have, for the most part, the following formula:



wherein:

T is H or a sulfur protecting group;

each X independently represents H_2 or O;

each R independently represents a substituent selected from the group consisting of hydrogen; alkyl; carboxylate; geminal dialkyl; a non-alkyl side chain of an amino acid other than cysteine (alkyl side chains being covered when R is an alkyl group); $-(CH_2)_n-COOH$; and $-(CH_2)_n-Z$;

Z represents a chemically reactive moiety capable of reacting with a recognition agent and binding the chelate thereto;

n is an integer of from 1 to about 4; and

R' is H_2; $-(CH_2)_n-COOH$; $-(CH_2)_n-Z$; or an alkyl group having one or more polar groups substituted thereon;

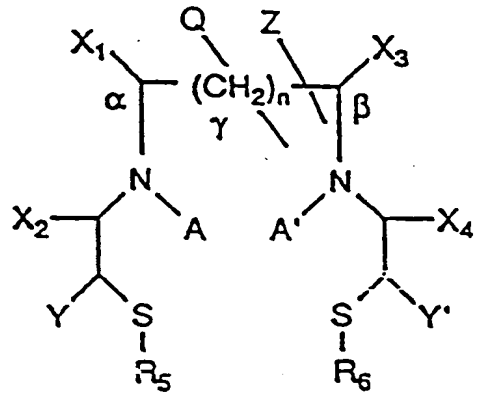
wherein the compound comprises at least one $-(CH_2)_n-Z$ substituent.

When Z is $-NH_2$, n should be at least 2. When Z is an ester and is at the R' position, n preferably is 3.

The sulfur protecting group may be selected from alkyl, aryl, acyl (preferably alkanoyl or benzoyl), thioacyl groups having 1 to about 7 carbons, and organothio groups having 1 to about 10 carbons.

For the R groups, the alkyl groups generally contain from 1 to 7 carbons, preferably from 1 to 4 carbons, and most preferably represent methyl.

The N_2S_3 and N_2S_4 chelating compounds have the following formula:



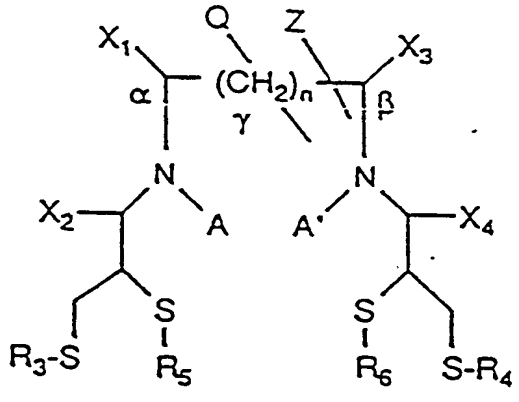
Examples of specific embodiments of the elements include the following:

X_1 and X_2 may be H or an oxy group (=0), but both are not =0. Likewise, X_3 and X_4 may be H or =0, but both are not =0. By selecting =0 for X_1 or X_2, the N interposed between the carbons to which X_1 and X_2, are bonded will be an amide. Likewise, by selecting =0 for X_3 or X_4, the N interposed between the carbons to which X_3 and X_4 are bonded will be an amide. Thus, a compound with zero, one or two amides may be formed by the appropriate selection of X_1, X_2, X_3 and X_4. Amide nitrogens, relative to amine nitrogens, afford greater stability to the complex formed with a metal, but at the expense of a diminished acceleration of complex formation. Thus, by selection of X_1, X_2, X_3 and X_4, compounds with a wide variety of chelating properties may be formed.

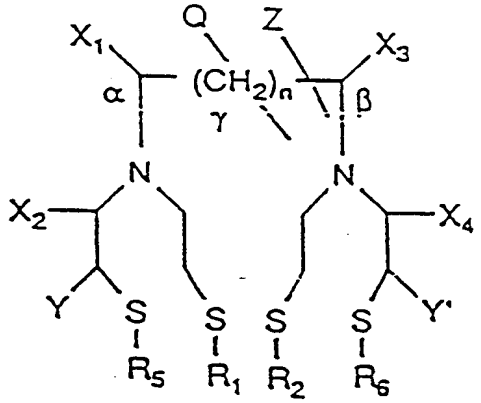
A is hydrogen (H), alkyl group of C_6 or less, $-CH_2-CH_2-S-R_1$ or $-CO-CH_2-S-R_1$, except when either X_1 or X_2 is =0, A is H. Similarly, A' is H, alkyl group of C_6 or

less, $-CH_2-CH_2-S-R_2$ or $-CO-CH_2-S-R_2$, except when either X_3 or X_4 is =0, A' is H.

Y is $-CH_2-S-R_3$, or H, when A is H or an alkyl group of C_6 or less and A' is H or an alkyl group of C_6 or less. Alternatively, when either A or A' or both are not H or an alkyl group of C_6 or less, then Y is H. Similarly, Y' is $-CH_2-S-R_4$, or H, when A is H or an alkyl group of C_6 or less and A' is H or an alkyl group of C_6 or less. Alternatively, when either A or A' or both are not H or an alkyl group of C_6 or less, then Y is H. However, Y and Y' are both not H when A is H or an alkyl group of C_6 or less and A' is H or an alkyl group of C_6 or less. Thus, compounds of the formula depicted above may be formed containing two nitrogens and three or four sulfurs ("N_2S_3" and "N_2S_4", respectively). For "N_2S_4" compounds, two of the sulfurs are the sulfurs bearing R_5 and R_6 and the remaining two sulfurs are from A and A' or Y and Y'. The following formulae depict examples of N_2S_4 compounds in which two sulfurs are from Y and Y' (A) or in which two sulfurs are from A and A' (B).



A



B

R_1, R_2, R_3, R_4, R_5 and R_6 are independently selected from sulfur protecting groups. Groups which may be used include any of the alkyl, acyl and aryl groups,

disulfides and bunte salts known by those skilled in the art.

Preferred groups are those that result in the formation of a thioacetal, hemithioacetal, thioester or acetamidomethyl substituent. Particularly preferred groups include p-anisylidine, acetonyl, tetrahydryl-furanyl, ethoxyethyl, tetrahydrylpyranyl, acetamidomethyl and derivatives thereof. When conjugated to a recognition agent, some of these protecting groups may be removed and left as sulfhydryls, either during storage or just prior to radiolabeling. With hemithioacetal protecting groups, removal prior to radiolabeling is unnecessary.

Q may be H or a polar group. One function of a polar group is to increase the hydrophilicity of the compound, e.g., to increase its aqueous solubility. Groups which may be used include carboxylates, sulfonates and secondary alcohols. A preferred group is $-CH_2-COOH$. Q may be attached to one of the positions designated as α, β, and gamma. Because the number of methylene carbons at the gamma position is defined by n, which may be greater than one, the gamma position includes additional points for attachment of Q.

The distance by which the nitrogen atoms are separated may be increased by interposing methylene ($-CH_2-$) groups between the carbons bonded to the nitrogens. When the number of $-CH_2-$ groups, represented by n, is greater than zero, then the number of carbon atoms separating the nitrogen atoms in compound I is increased accordingly. Preferred integers for n are 0 to 2.

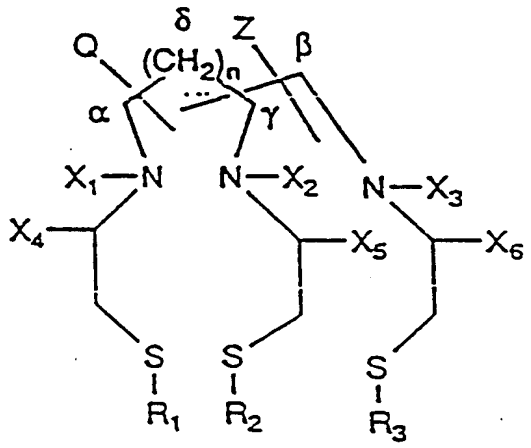
Z is $-(W)_m-R'$. W is a group that functions as a "spacer arm" and may be useful to distance R' from the chelating portion of the compound. Groups which may be used include methylene ($-CH_2-$), methyleneoxy ($-CH_2-O-$), methylenecarbonyl ($-CH_2-CO-$), or combinations thereof.

The number, m, of groups such as these would be typically 0 to about 30 and preferably 0 to about 5.

Z, or R' when m is 0, may be attached to one of the positions designated as α, β, and gamma. Because the number of methylene carbons at the gamma position is defined by n, which may be greater than one, the gamma position includes additional points for attachment of a Z or an R'.

R' is a chemically reactive moiety capable of reacting with a recognition agent and binding the chelate thereto.

N_3S_3 compounds which contain three nitrogens and three sulfurs, have the following formula:



Examples of specific embodiments of the elements include the following.

R_1, R_2, and R_3 are independently selected from sulfur protecting groups. Groups which may be used include any of the alkyl, acyl, aryl groups, disulfides and bunte salts known by those skilled in the art. Preferred groups are those that result in an acyl, a thioacetal or a hemithioacetal. Particularly preferred groups include thioesters, p-anisylidine, acetonyl, tetrahydrylfuranyl, ethoxyethyl, tetrahydrylpyranyl, acetamidomethyl, and derivatives thereof.

X_1 and X_2 are independently selected from hydrogen and an alkyl group of C_6 or less. X_3 is H, an alkyl group of C_6 or less, or Z. X_4, X_5, and X_6 are independently selected from H and =0. The selection of =0 results in the presence of an amide. Thus, a compound with zero, one, two or three amides may be formed by the appropriate selection of X_4, X_5, and X_6. Amide nitrogens, relative to amine nitrogens, afford greater stability to the complex formed with a metal, but at the expense of a diminished acceleration of complex formation. Thus, by selection of X_4, X_5, and X_6, compounds with a wide variety of chelating properties may be formed.

Q may be H or a polar group. One function of a polar group is to increase the hydrophilicity of the compound, e.g., to increase it aqueous solubility. Groups which may be used include carboxylates, sulfonates and secondary alcohols. A preferred group is $-CH_2-COOH$. Q may be attached to one of the positions designated as α, β, gamma, and δ. Because the number of methylene carbons at the δ position is defined by n, which may be greater than one, the δ position includes additional points for attachment of Q.

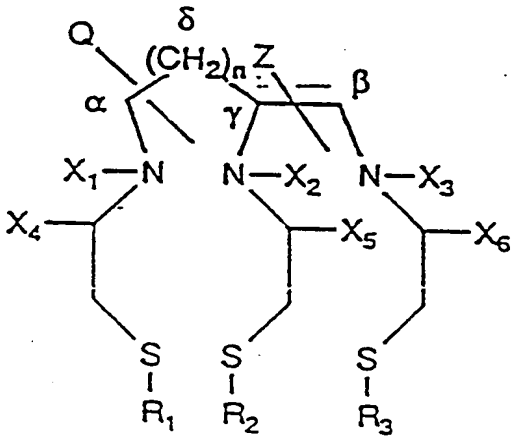
The distance by which the nitrogen atoms are separated may be increased by interposing methylene ($-CH_2-$) groups between the carbons bonded to the nitrogens. When the number of $-CH_2-$ groups, represented by n, is greater than zero, then the number of carbon atoms separating the nitrogen atoms in compound II is increased accordingly. Preferred integers for n are 0 to 4.

Z is $-(W)_m-R'$. W is a group that functions as a "spacer arm" and may be useful to distance R' from the chelating portion of the compound. Groups which may be used include methylene ($-CH_2-$), methyleneoxy ($-CH_2-O-$), methylenecarbonyl ($-CH_2-CO-$), or combinations thereof. The number, m, of groups such as these would be typically 0 to about 30 and preferably 0 to about 5.

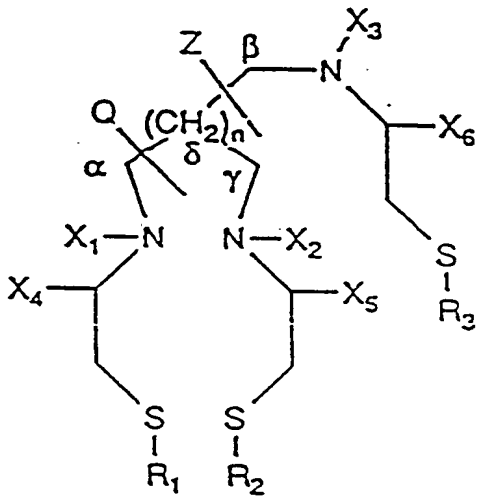
Z, or R' when m is 0, may be attached to X_3 or to one of the positions designated as α, β, gamma, and δ. Because the number of methylene carbons at the δ position is defined by n, which may be greater than one, the δ position includes additional points for attachment of a Z or an R'.

R' is a chemically reactive group capable of reacting with a recognition agent and binding the chelate thereto.

In N_3S_3 compounds, the carbon designated as β may be bonded to any one of the carbons designated as α, gamma and δ. The following formulae depict compounds in which the β carbon is bonded to the gamma carbon (A) and the β carbon is bonded to the δ carbon (B).



A



B

The chelating compounds of the present invention containing four to six donor sulfur and nitrogen atoms may be obtained in a manner described in European Patent Application Publication No. 0188256.

In the context of the present invention, the term "chemically reactive group" refers to a functional moiety capable of reacting with a recognition agent and thereby binding the chelate to that recognition agent. This

chemically reactive group may be strongly electrophilic or nucleophilic, and thereby be available for reacting directly with a recognition agent. Alternatively, the moiety may be a weaker electrophile or nucleophile, and therefore require activation prior to binding with a recognition agent. This alternative would be desirable where it is necessary to delay activation of the chemically reactive moiety until a compound has been formed.

In either scenario, the chemically reactive moiety designated by various letter symbols in the formulae is, indeed, chemically reactive. The scenarios differ by whether, following formation of a compound, the chemically reactive group is sufficiently reactive to be reacted directly with a recognition agent, or is activated first with one or more chemicals to render the group capable of reaction with a recognition agent. Illustrative chemically reactive groups and reactions thereof are described below.

Three methods are provided for producing the chelate-recognition conjugate useful in the method of the present invention. The first method features binding of the recognition agent to a radiolabeled compound, e.g., after a radiometal or radiometal oxide has been added to a chelating compound. A second method involves binding of the recognition agent to a fully formed, yet unlabeled, chelating compound, e.g., prior to the addition of a radiometal or radiometal oxide to the chelating compound. In both instances, the recognition agent is bound to the chelating compound via a chemically reactive group.

The step of combining a recognition agent with a labeled or unlabeled compound may be performed by direct reaction of the recognition agent with a chemically reactive moiety. This combination can also be achieved by "direct" reaction of a pre-activated chemically reactive moi ty, as described above with a recognition

agent. Alternatively, it may be desirable to include a preparatory step prior to the combining step to enhance the binding capability of the recognition agent. Such modification of the recognition agent may include reaction with any of the numerous bifunctional reagents reported in the literature.

A direct reaction involving a chelating compound and a modified or unmodified recognition agent requires a chemically reactive moiety capable of reacting with the modified or unmodified recognition agent. Exemplary chemically reactive moieties useful in the present invention include an alkyl group containing a good leaving group such as a halide, or a carbonyl-containing group such as an anhydride, an acid halide or an "active ester".

By an "active ester", there is contemplated esters that are highly reactive in nucleophilic substitution reactions. In the present invention, the modified or unmodified recognition agent would serve as the nucleophile. Typically, the esters will be activated phenols and cyclic compounds based upon hydroxylamine. Examples of commonly used "active" ester groups are tetrafluorophenyl, N-succinimidyl, nitrophenyl, isothiocyanate and substituted isothiocyanates. Alternatively, a chemically reactive moiety may serve as the nucleophile, such as an amino or sulfhydryl group capable of reacting with a modified recognition agent, e.g., a recognition agent containing a maleimide moiety.

Another preparatory step optionally used in the practice of the present invention is the activation of the chemically reactive moiety to enhance reactivity of the chelating compound with the recognition agent, as referred to above. Exemplary of such an activation is the conversion of a carboxyl moiety into an active ester. Another example is the activation of a chemical reactive moiety protected by a protective group. Removal of the

protective group constitutes an activation. For example, removal of a phenylsulfonyl protective group from a succinimide derivative results in the conversion of the succinimide moiety into a maleimide moiety, which is highly reactive in nucleophilic addition reactions. Activation of the chemically reactive moiety also includes reaction of a nucleophilic moiety on the chelating compound with a bifunctional reagent. It will be evident to one skilled in the art that a variety of homobifunctional and heterobifunctional agents may be employed within the present invention to achieve such activation.

A third method for providing a radiolabeled recognition agent using a chelating compound bridge incorporates into the recognition agent a compound that is suitable for radiolabeling during the synthesis of such a compound. That is, a recognition agent is covalently attached to a precursor of a compound suitable for radiolabeling. Following this covalent attachment, the synthesis of the precursor compound is completed, such that the resultant chelating compound-recognition agent complex is suitable for radiolabeling.

As a recognition agent useful in the present invention, there is contemplated a monoclonal antibody or fragment thereof directed against a leukocyte activation marker. A leukocyte activation marker is a cell surface antigen which is poorly expressed or not expressed at all on leukocytes until the leukocyte is activated or caused to differentiate.

Activation of leukocytes, such as PMNs and monocytes, and their migration to sites of inflammation appear to take place in vivo as a result of an inflammatory response. Granulocyte activation may also be induced ex vivo by treatment with activators, such as granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF),

gamma interferon, a calcium ionophore or by other agents capable of inducing an oxidative burst. Similarly, monocytes can be activated with gamma-interferon, monocyte colony stimulating factor (M-CSF), colony stimulating factor-1 (CSF-1) or tumor necrosis factor (TNF). Natural killer (NK) cells can be activated with alpha or gamma-interferon and interleukin-2, and T-cells may be activated by interleukin-2, interleukin-4 and other interleukins.

Preferably, the recognition agent is a monoclonal antibody or fragment thereof directed against an epitope of a leukocyte-associated antigen which is only expressed after activation, or which exhibits enhanced cell surface expression following activation. A useful activation marker within the present invention is one associated with a leukocyte surface antigen involved in chemotaxis, phagocytosis or cell killing which are functions normally enhanced with respect to activated leukocytes.

Exemplary epitopes useful as activation markers include epitopes associated with the lymphocyte function associated antigens (LFA-1, LFA-2 and LFA-3), LEU-CAM, CD2, the LFA ligand, complement receptors CR1 and CR3, Fc receptors (I, II, III), a leukotriene receptor or a chemotactic factor receptor. Preferable chemotactic factor receptors recognize and bind C5a, C3a and formyl-methionine-leucine-phenylalanine (fMLF). Complement receptors include those for C1q and C3 fragments.

Recognition agents useful in the present invention selectively interact with activated leukocytes. That is, recognition agents of the present invention exhibit at least a 10-fold preference for binding to activated leukocytes over binding to non-activated leukocytes. For example, leukocyte receptor inhibitors are useful as recognition agents within the present invention.

The relationship between these antigens/epitopes and useful recognition agents may be further elucidated by

way of example. As mentioned above, the C3 receptor is up-regulated upon leukocyte activation. As a result, monoclonal antibodies or fragments thereof specific for CR3 may be used as recognition agents. Normally, the expression of such receptor is below 10^3 sites/cell and, therefore, insufficient for imaging. Up-regulation upon activation would both increase receptor number (making the receptor suitable for imaging) and increase receptor affinity (making it appropriate for imaging with labeled ligand). Thus, a ligand like a complement fragment may be labeled and used as a recognition agent in the practice of the present invention. Other exemplary ligands for up-regulated receptors include chemotactic peptides like fMLF, peptides derived from C3a and C5a capable of binding their respective receptors, immunoglobulin Fc peptides capable of binding Fc receptors, and complement components like C1q or C3 fragments capable of binding their respective receptors.

An embodiment of the present invention features a monoclonal antibody or fragment thereof as a recognition agent directed against complement fragments that are bound at inflamed tissue sites or adsorbed to activated leukocyte receptors. A preferred embodiment of the present invention involves the use of a monoclonal antibody directed against C3dg. C3dg is an especially useful target for several reasons. First, C3dg is a cell- or tissue-bound activation product of the complement cascade, and will be present as a result of complement activation through the classical or alternative pathways. Second, since C3dg is the final degradation product of C3, antibodies directed to C3dg will assuredly react with tissue sites of activation, as opposed to antibodies directed to C3, C3b, or iC3b, which may react to determinants lost with further degradation. Third, antibodies to C3dg will be more sensitive in detecting inflammation than antibodies to other complement compo-

nents, since C3 activation represents the point of amplification in the complement cascade. For instance, for each molecule of C1q bound to cells or tissues, 100 molecules of C3b are bound. Fourth, antibodies to C3dg would have high selectivity for sites of inflammation similar to other antibodies to C3 fragments, since the presence of C3dg at any tissue site would require three different proteolytic cleavage steps, each regulated by a variety of mechanisms.

A further embodiment of the present invention involves the use of a monoclonal antibody or fragment thereof directed against activated leukocytes that does not exhibit substantial interaction with non-activated leukocytes. Use of this type of monoclonal antibody permits a qualitative (rather than merely quantitative) distinction to be made between activated and non-activated leukocytes by the label-recognition agent conjugate.

A preferred embodiment of the present invention involves the use of a conformation-dependent determinant on a cell surface lymphoid activation marker. The specificity of such conformation-dependent determinant is associated with leukocyte adhesion or aggregation that may occur when an activated leukocyte contacts vascular endothelium, undergoes phagocytosis of a microorganism or adheres to a target cell, as in a cytolytic process. For example, vascular endothelium expresses an adhesion protein designated I-CAM. I-CAM interacts with PMN surface LFA antigens to cause adherence of PMNs to the vascular endothelium. When an activated PMN undergoes adhesion to vascular endothelium, conformational changes occur in leukocyte membrane proteins that contact the vascular endothelium. Since these unique conformation-dependent epitopes are exposed only upon adhesion of PMNs to the vascular endothelium, an antibody directed at said epitopes will only recognize PMNs at sites of inflammation.

This LFA/I-CAM adherence is markedly increased by prior activation of PMNs which up-regulates LFA expression.

Similar determinants are exposed upon homotypic aggregation of activated leukocytic cells. Moreover, PMNs express a marker that is substantially equivalent to I-CAM. This determinant is designated LEU-CAM and is capable of interacting with LFA in furtherance of homotypic aggregation of PMNs. Such homotypic aggregation, thus appears to be LFA and LEU-CAM mediated. As a result, a monoclonal antibody directed to conformation-dependent epitopes expressed by homotypic aggregates of leukocytes but not non-aggregated leukocytes will exhibit specificity for activated leukocytes.

Monoclonal antibodies or fragments thereof of the present invention may be prepared according to conventional techniques. See, for example, Kohler and Milstein (1975, Nature 256: 495-97; 1976, Eur. J. Immunol. 6: 511-519). Antibodies to conformation-dependent determinants or activation markers are generated, for example, by immunizing mice with homotypic aggregates of activated PMNs, monocytes, cultured myelomonocytic cell lines (such as U-937 or THP-1), or with other suitable cell sources bearing leukocyte markers like LFA. In a preferred embodiment, mice are immunized with PMNs that are pooled from normal donors and activated with GM-CSF in the presence of human serum until aggregation occurs. Activation of PMNs allows for up-regulation of LFA, complement and chemotactic peptide receptors, as well as the adsorption of complement components to activated cells. Hybridomas generated by the immunogen are then screened against the original immunizing cells adsorbed to poly-L-lysine-coated microtiter plates or against non-activated pooled PMNs in the presence of human serum. Desired antibodies will recognize activation markers as well as conformation dependent determinants. The monoclonal antibodies can then be further screened against

inflammatory lesions by using immunoperoxidase techniques.

Eosinophilotactic peptides may also be used as recognition agents within the present invention. Exemplary peptides are VGDE (val-gly-asp-glu), VGSE (val-gly-ser-glu), VGAE (val-gly-ala-glu) and AGSE (ala-gly-ser-glu). These exemplary peptides are described in PNAS 72: 4123 (1975), Immunology 32: 57 (1977) and Clinical Experimental Immunology, 43: 399 (1981). Eosinophilotactic peptides may be labeled through conventional techniques, preferably through the attachment of a spacer portion having a terminal tyrosine residue (the tyrosine residue may, of course, be attached after the spacer portion has been bound to the peptide). The tyrosine residue may then be radiolabeled.

As another recognition agent of the present invention, there is contemplated a chemotactic factor. A chemotactic factor is a factor that attracts polymorphonuclear leukocytes through a process called chemotaxis. Exemplary chemotactic factors useful in the present invention include chemotactic peptides such as fMLF and peptides of complement proteins, fragments thereof or derivatives or analogs thereof. Longer chemotactic peptides, such as fibrinopeptide B (pyroglu-G-V-N-D-N-E-E-G-F-F-S-A-R), may also be used in accordance with the present invention. Preferred complement proteins useful as recognition agents are C3a and C5a, fragments thereof or derivatives or analogs thereof. Exemplary analogs are:

f-norLeu-L-F-norLeu-Y-K, wherein the tyrosine (Y) residue may be radiolabeled directly; and

f-norLeu-L-F-norLeu-;

boc-L-F-L-F-;

boc-M-L-F-

boc-F-L-F-L-F-;

boc-F-L_b-F-L_b-F-; and

f-M-L-Y-, wherein boc is T-butoxycarbonyl, L_D is D-Leu and wherein the analogs may be labeled through conv n-tional techniques, preferably through the attachment of a spacer portion having a terminal tyrosine residue (the tyrosine residue may, of course, be attached after the spacer portion has been bound to the peptide). The tyrosine residue may then be radiolabeled. These exemplary peptide analogs are described in Science 205: 1412 (1979), Biochim. Biophys. Acta 602: 285 (1980), Nature 272: 462 (1978), PNAS 73: 2439 (1976) and Biochem. Biophys. Res. Comm. 30: 464 (1978).

Also useful as recognition agents in the practice of the present invention are chemotactic peptide receptor inhibitors. Such inhibitors will bind to the chemotactic peptide receptor with high affinity. Peptides of complement protein C3a des Arg are also useful as recognition agents.

Chemotactic peptides may be prepared synthetically by conventional techniques. One embodiment of the label-recognition agent conjugate of the present invention involves the incorporation of D-amino acid(s) into a synthetic peptide chain, thereby decreasing in vivo degradation of the synthetic peptide. Degradative processes of the host recognize naturally-occurring L-amino acids. Thus, incorporation of one or more D-amino acids into the synthetic peptide enhances the stability of the peptide in vivo.

Another embodiment of the imaging method of the present invention as related to small synthetic peptides used as recognition agents involves the additional steps of:

(4) comparing inflammation site localization of the chemotactic peptide and reticuloendothelial system localization of the peptide, wherein exhibition of a substantial affinity of the peptide for circulating or

reticuloendothelial cells necessitates step (5); and, if necessary,

(5) altering the amino acid sequence of the peptide through addition or deletion of amino acids, to more closely correlate the peptide structure with that bound by high affinity receptors of the activated leukocytes and less closely correlate with that bound by receptors of non-activated cells,

thereby producing a modified peptide capable of preferentially binding to activated leukocytes at sites of inflammation.

Labeled peptide can be compared for binding to activated and non-activated cells. Modified or non-modified peptides are screened against activated and non-activated PMN or monocytes for binding. A comparison of the difference in selectivity of peptides bound to activated and non-activated cells at different concentrations will indicate relative specificity of the peptide for activated and non-activated cells.

The comparison of step (4) may also be accomplished through analysis of an image obtained as described in steps (1)-(3). Such analysis is within the ordinary skill of a diagnostician familiar with diagnostic imaging of this type.

The alteration of step (5) may be accomplished by conventional protein synthetic techniques, following analysis of the binding to activated and non-activated cells. Activated leukocytes express receptors of higher affinity than non-activated leukocytes. Thus, recognition agents may be modified to more specifically associate with these high affinity receptors. Such alteration of recognition agents may be steric or chemical. That is, the change can serve to improve the steric "fit", i.e., the actual three-dimensional structural congruence of the peptide and the target leukocytes, or to improve the chemical "fit", i.e., the cor-

respondence of positively and negatively charged amino acids between the peptide and the target leukocyte receptor.

Enhanced target cell label retention, may be obtained by substituting longer, more hydrophobic or charged amino acids to the chemotactic protein. These modified peptides may enhance the accumulation of label at target tissue sites by increasing the peptide's ability to anchor to target cells, for example, by incorporation of a "spacer" amino acid portion to increase the length of the targeting peptide thereby increasing the accessibility of the peptide binding site to appropriate receptors on target cells.

Additional hydrophobic or like-charged polar "spacer" amino acid sequences can also be used to enhance peptide access to a receptor. For example, a chain of glycines (poly-G) could be added to the carboxy terminus of fMLF to increase chain length. A plurality of alanines (poly-A) may be added to produce a longer, more hydrophobic moiety. Aspartic acid (D) residues can be added to obtain a longer, negatively charged recognition agent while arginine (R) residues will impart positive charge as well as increased length. In each case, it may be necessary to include another amino acid, such as cysteine, to permit binding of the modified chemotactic peptide to a chelating compound.

Another procedure that may alter the serum half-life of the peptide (e.g., deliver an increased percentage of dose/gram to site of inflammation) and increase affinity of the peptide for the target is conjugation of the peptide or peptide-spacer to a macromolecular carrier, such as albumin.

Radiolabeling of antibodies and proteins may be accomplished through known techniques, such as those described in European Patent Application Publication Nos. 0188256, 0289187 and 0203764. Alternatively, smaller,

synthetically prepared peptides, such as fMLF, may be labeled by other techniques. For example, a synthetic peptide may be radiolabeled via tyrosine, lysine or cysteine or phenylalanine residue or analog thereof added to the peptide during conventional protein synthesis. Labeling such synthetic peptides may be done, for example, in two steps. First, the peptide having an additional residue is synthesized. Second, the residue is labeled by known techniques such as linking via a hetero bi-functional chelate.

Within the present invention, the labeled recognition agent is infused into a patient whose tissue sites are to be imaged. This infusion may be conducted in any manner adequate to deliver the labeled recognition agent to the bloodstream of the patient. Exemplary of acceptable administration routes are intraperitoneal, subcutaneous, intradermal, intraarterial or intravenous injection. The mode of administration is typically chosen according to the projected ultimate destination of the labeled recognition agent. Such infusions may be given as single or multiple injections. The labeled recognition agent may be administered through injection directly into the damaged tissue location, where convenient.

In vivo administration of labeled recognition agent may involve the use of pharmaceutical compositions in which the labeled recognition agent is dispersed in a pharmaceutically acceptable carrier. Exemplary of such pharmaceutically acceptable carrier is physiological saline or a physiologically acceptable buffer solution.

Generally, the amount of the labeled recognition agent administered to a patient will depend primarily on the size of the patient and the purpose of the administration. However, the patient's physiological condition and the tissue site to be imaged or treated, if known, may affect the amount of labeled recognition agent neces-

sary to obtain a usable image. Dosage of labeled recognition agent may readily be determined by one of ordinary skill in diagnostic imaging. A typical dose of radiolabeled recognition agent is between about 1 and about 3000 mCi. In humans, a standard imaging dose will be from about 1 to about 50 mCi, with about 10 to about 30 mCi being typical.

The imaging of the present invention may be accomplished with the aid of one of the many commercially available imaging cameras, such as the Picker Digital Dynascan camera, the Raytheon LFOV Anger gamma camera and the gamma camera STARCAM made by General Electric Corporation. Visualization of sites of inflammation may be obtained by planar or single photon emission computed tomographic (SPECT) scans.

The time lapse between infusion of the labeled recognition agent and scan or imaging will vary somewhat with the patient's characteristics, i.e., body weight and condition, as well as the administration route, recognition agent and label used. Typically, a lapse of between 3 and 144 hours is required to allow the labeled recognition agent the opportunity to migrate to the target tissue and clear from uninvolved tissue. An appropriate time lapse is readily determinable by a person ordinarily skilled in diagnostic imaging.

Images produced according to the present invention may aid in the detection of tissue damage mediated by inflammation. A diagnostician will recognize image patterns characterizing such an ailment. Also, the images produced according to the present invention will provide the diagnostician with information regarding the extent of tissue damage or area susceptible to tissue damage, as with myocardial infarction. Also, a sequence of images of an afflicted tissue site will permit monitoring of treatment protocols designed to alleviat inflammation.

Another aspect of the present invention involves a method of imaging a tissue site of inflammation including:

(1) infusing non-labeled recognition agent into a patient, wherein the agent is capable of interacting with activated leukocytes accumulated at the tissue site;

(2) labeling a recognition agent;

(3) infusing labeled recognition agent into the patient; and

(4) imaging said tissue site,

whereby medical conditions involving tissue inflammation may be detected, evaluated and monitored. As can be readily appreciated, the exact ordering of steps (1) and (2) may be altered without materially altering the procedure.

This embodiment of the present invention addresses the problem of accumulation of labeled recognition agent within the reticuloendothelial system or on circulating cells. Accumulation in the RES will decrease the clarity of the image by creating "visual background".

That is, if normal tissue expression of the antigen or determinant recognized by the labeled antibody is more accessible to recognition agent than the inflamed tissue site of the antigen or determinant, normal tissue sites may be saturated before the inflamed tissue sites are filled. See, for example, co-pending United States patent application, serial number 917,176.

For example, antigen-bearing normal cells within the bloodstream will be more accessible to "cold" antibody than non-circulating inflammatory tissue cells. Thus, "cold" agent will associate preferentially with the more accessible, peripheral, antigen-bearing normal cells. As a result, subsequently infused labeled or "hot" antibody will be more likely to reach and bind the less accessible, antigen-bearing inflammatory tissue cells. If a significant, accessible normal tissue

antigen pool does not exist, no pre-infusion of "cold" agent is necessary.

For "cold" infusion, a non-labeled recognition agent is infused into a patient whose tissue sites are to be treated in the same or different manner than with the labeled recognition agent. In vivo administration of non-labeled recognition agent may involve the use of pharmaceutical compositions in which dispersion in a pharmaceutically acceptable carrier is necessary or desirable. Exemplary of such a pharmaceutically acceptable carrier is physiological saline or a physiologically acceptable buffer solution.

Generally, the amount of non-labeled recognition agent administered to a patient will depend primarily on the size of the patient. However, the patient's physiological condition and the tissue site to be imaged, if known, may affect the amount of non-labeled recognition agent required to obtain a diagnostic image substantially free of background. Dosage of non-labeled recognition agent may readily be determined by one of ordinary skill in diagnostic imaging. Saturation of peripheral cell sites may be monitored by removing samples of circulating cells and measuring percent saturation by flow cytometry.

The time lapse between infusion of non-labeled recognition agent and labeled recognition agent will vary somewhat with the patient's characteristics (i.e., body weight and condition), as well as with the administration route, recognition agent and label used. The time lapse necessary to allow the non-labeled recognition agent adequate opportunity to associate with normal cells is readily determinable by a person ordinarily skilled in diagnostic imaging.

In this aspect of the present invention, the "hot" recognition agent need not exhibit marked specificity for activated over non-activated leukocytes since the "cold" recognition agent will bind sites located on the more

easily accessible peripheral leukocytes. However, further enhancement of label at target tissue sites can be attained if the "hot" recognition agent binds preferentially to activated leukocytes.

When synthetic peptides are used as recognition agents, a second level of reduction in visual background is possible regarding images produced by the invention. The method may be performed as above with the further steps of:

(5) comparing inflammation site localization of the chemotactic peptide and reticuloendothelial system localization of the peptide, wherein exhibition of a substantial affinity of the peptide for circulating or reticuloendothelial cells necessitates step (6); and, if necessary,

(6) altering the amino acid sequence of the peptide through addition or deletion of amino acids, so as to more closely correlate the peptide structure with that bound by high affinity receptors of the activated leukocytes and less closely correlate with that bound by receptors of non-activated cells, thereby producing a modified peptide capable of preferentially binding to activated leukocytes at sites of inflammation.

The first and second aspects of the present invention described above involves the <u>in vivo</u> association of the labeled recognition agent with target leukocyte cells. The third aspect of the present invention involves <u>ex vivo</u> association of recognition agent with leukocyte cells. <u>In vivo</u> association techniques may also be used in conjunction with the <u>ex vivo</u> association within the second aspect of the present invention.

Specifically, the third aspect of the present invention contemplates a method of imaging a tissue site of inflammation involving:

(1) labeling a recognition agent, wherein the agent is capable of interacting with a leukocyte binding moiety;

(2) withdrawing leukocytes from a patient;

(3) incubating leukocytes of step (2) with labeled recognition agent of step (1);

(4) infusing into the patient labeled recognition agent and leukocytes incubated in step (3); and

(5) imaging the tissue site,

whereby medical conditions involving tissue inflammation may be detected, evaluated and monitored. As can be readily appreciated, the exact ordering of steps, most notably steps (1) and (2) may be altered without materially altering the procedure.

The method of this aspect of the present invention may further include an activation step following withdrawal step (2). That is, the withdrawn leukocytes can optionally be activated by incubation with an activation agent as previously described or by other means suitable to accomplish such activation.

Exemplary leukocyte binding moieties of the present invention are chemotactic peptide receptors. These receptors and recognition agents useful in targeting such receptors have been previously discussed.

Complement receptors are also useful leukocyte binding moieties within the third aspect of the present invention. C3a and C5a receptors are especially useful in the practice of the present invention. The corresponding complement components or analogs or derivatives thereof may be used as recognition agents within these embodiments of the present invention.

An additional leukocyte binding moiety is a leukocyte surface antigen which up-regulates upon leukocyte activation. In this embodiment, Fab or $F(ab')_2$ fragments of monoclonal antibodies capable of recognizing an up-

regulated leukocyte surface antigen can be used as recognition agents.

Another exemplary leukocyte binding moiety of the present invention is an adhesion protein. Adhesion proteins are those which are involved in the adhesion of leukocytes to each other or other cells. Exemplary adhesion proteins are LFA-1, LFA-2 and LFA-3.

Additionally, adhesion protein receptors are useful as leukocyte binding moieties in the practice of this aspect of the present invention. Exemplary adhesion protein receptors are located, for example, on the vascular endothelium (I-CAM) or on the leukocytes themselves (LEU-CAM).

This aspect of the present invention may be described in the following manner. Labeled recognition agent directed to a receptor for adhesion proteins is incubated together with activated or non-activated, autologous PMNs or monocytes followed by reinfusion into the host.

Labeled leukocytes will accumulate at sites of inflammation. This represents an improvement over prior art processes which involved either oxidative cell surface labeling or incubation of leukocytes with whole antibody specific to non-activation markers.

When non-activated leukocytes are used in incubation step (3), accumulation of label at target tissue sites will be enhanced due to migration of reinfused autologous leukocytes associated with recognition agent-label conjugates to the target tissue site as well as by virtue of the specificity of the recognition agent for activated leukocytes located at the target site.

In the use of activated leukocytes in incubation step (3), label localization improves due to an increase in activation markers. However, coincident with this up-regulation, LFA expression is enhanced. Enhanced LFA expression leads to increased interaction with I-CAM

sequences on vascular endothelium and reduced extravasation of activated PMNs into tissue. Enhanced LFA expression also results in increased aggregation with LEU-CAM sequences on other activated leukocytes and homotypic aggregation of those activated leukocytes.

In addition to antibody to LFA, peptides containing RGDS (arginine-glycine-aspartic acid-serine) sequences or other "I-CAM like" sequences, can be used as the labeled recognition agent. By "I-CAM like" sequences, there are contemplated sequences which are substantially functionally equivalent to I-CAM. For the purposes of this invention, LEU-CAM is a substantially functional equivalent of I-CAM.

Thus, in an example of this aspect of the present invention, PMNs are activated by GM-CSF or one or more other activation agents, and then incubated with labeled Fab or $F(ab')_2$ fragments of an antibody to LFA capable of inhibiting interaction with I-CAM, such as the antibodies 4F-2 and OKM-1. Since receptors for chemotactic stimuli are unblocked, cells can still chemotax efficiently and extravasate. As anti-LFA antibody fragment is lost from the cell surface of PMNs, cells which have extravasated into sites of inflammation will be blocked from re-egress into the circulation.

In a modification of this aspect, Fab or $F(ab')_2$ to LFA can be infused upon reinjection of labeled, activated autologous PMNs, to maintain the blockade of LFA interaction with I-CAM and to ensure that cells do not accumulate in the RES.

An additional embodiment of the present invention features a method of imaging a tissue site of inflammation including:

(1) withdrawing leukocytes from a patient;

(2) constructing a chemotactic peptide or a fragment, a derivative or analog thereof containing an affinity label and a radionuclide label;

(3) incubating leukocytes of step (1) with the peptide of step (2) for a time sufficient to permit binding thereof;

(4) photoactivating said photoaffinity label;

(5) infusing into the patient labeled peptide and leukocytes resulting from step (4); and

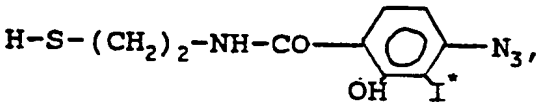
(6) imaging the tissue site,

whereby medical conditions involving tissue inflammation may be detected, evaluated and monitored.

Affinity labeling, such as photoaffinity labeling, allows for specific covalent labeling of cells. That is, the peptide is non-covalently bound to a leukocyte under conditions which will not photoactivate the peptide, i.e., without exposure of the incubation mixture to light of the relevant wavelength. Upon photoactivation by exposure to light as avoided previously, the affinity peptide will form an active moiety capable of insertion, for example, into carbon-hydrogen bonds such as those located on the leukocyte surface. As a result, the label will not be reversibly bound. Decreased reversibility of binding results in enhanced retention of the label at the target tissue site, which, in turn, permits more time for imaging. Enhanced label retention also results in a reduction in imaging background stemming from desorbed peptide. Affinity labelling in accordance with the present invention may be accomplished by standard techniques.

Exemplary chemotactic peptides which may be labeled by affinity methods are of the formulae f-M-L-F-spacer-Y, f-M-L-F-spacer-C or f-M-L-F-spacer-K. The spacer moiety may be any convenient small molecule, preferably a peptide, which does not interfere with the biological activity of the affinity labeled peptide. A chain of 1-5 glycines may be used as a spacer, with 1-2 glycine spacers preferred.

An exemplary affinity label is p-benzoyl-L-phe. Such a label is described in J. Biol. Chem. 261: 10695 (1986). Another exemplary affinity label is of the formula:



which is described in Biochem. Biophys. Acta 882: 271-80 (1986). A further exemplary affinity label is N-(4-(4'-azido-3'-[^{125}I]iodophenylazo)benzoyl)-N'-hydroxy-succinimide as described in PNAS 83: 5634-38 (1986).

The chemotactic portion of the photoaffinity stabilized construct of this aspect of the present invention may be additionally labeled with a radionuclide via an additional tyrosine moiety separated from the chemotactic peptide by a spacer portion, as is the first example of the chemotactic peptide shown above. This radionuclide serves as additional imaging input in the practice of the present invention. Alternatively, the photoaffinity label portion of the construct of this aspect of the present invention may be radionuclide labeled, as are the latter two examples of the photo-affinity portion noted above. Such radionuclide labeling may be accomplished by standard techniques as described above.

An application of the present invention involves diagnostic kits useful for in vivo imaging of tissue sites of inflammation comprising:

(1) recognition agent;

(2) instructions for labeling and administering the agent in a manner and amount sufficient to permit a diagnostic image to be obtained from tissue of the patient.

As a diagnostic kit, there is contemplated a collecti n of materials within a box or other container that are capable of being used in the present invention

with little additional processing by the end user. Such an end user need only provide items which are typically available to the practicing end user, such as imaging equipment, radiolabel and possibly some portion of the equipment necessary for administration of the agent to the patient. For example, sterile vials capable of use with standard syringes could be employed as containers for lyophilized recognition agent or solutions containing the recognition agent dispersed in a physiologically acceptable liquid. When lyophilized recognition agent is used, a sterile vial containing a physiologically acceptable buffer solution may be included in the diagnostic kit. The instructions for use of the diagnostic kit may be affixed to the container or be included as a separate insert within the container or both.

An embodiment of this aspect of the present invention involves a diagnostic kit, as previously described, which also contains an additional amount of recognition agent for ultimate use as non-labeled recognition agent. That is, a second sterile vial containing lyophilized recognition agent or a solution of recognition agent in a physiologically acceptable liquid is provided. When lyophilized recognition agent is used, an additional amount of physiologically acceptable buffer may be included in the kit.

Also, the present invention contemplates diagnostic kits useful for <u>in vivo</u> imaging of an ischemic tissue site in a patient suffering from a condition characterized by transient decrease in blood flow to tissue sites comprising:

(1) recognition agent;

(2) instructions for labeling and administering the agent in a manner and amount sufficient to permit a diagnostic image to be obtained from ischemic tissue of the patient.

An embodiment of this aspect of the present invention involves a diagnostic kit, as previously described, which also contains an additional amount of recognition agent for ultimate use as non-labeled recognition agent.

A decrease in blood flow to a tissue site causes a temporary deficiency of oxygen in that tissue. An inadequate oxygen supply results in tissue damage to deprived tissue sites. Cell death and release of mitochondrial proteins capable of binding C1q (the first component of the complement cascade) occur due to inadequate oxygen supply. Subsequent activation of the complement cascade and initiation of cellular infiltration (primarily by PMNs) result in further damage to cells.

For example, one of the earliest manifestations of myocardial infarction is ischemia associated with a reduction in blood flow to the heart muscle due to occluding clot formation. Ischemia, even if only transient, results in death of some cells and release of mitochondrial proteins capable of binding to C1q. This event leads to activation of the complement cascade and cellular infiltration which, in turn, result in additional cellular damage and infarct formation. Thus, ischemic heart muscle may be imaged in accordance with the present invention prior to the onset of myocardial infarction with its attendant inflammation. Ischemic heart muscle, hypoxic bowel tissue, vascular collagen diseased tissue, and tissue sites afflicted with cancer and characterized by decreased blood flow are exemplary of ischemic tissue sites.

Another application of the present invention involves a method of detecting a tissue site of inflammation in a patient comprising:

(1) labeling a recognition agent, wherein the agent is capable of interacting with activated leukocytes accumulated at the tissue site;

(2) infusing labeled recognition agent into the patient;

(3) imaging the tissue site; and

(4) analyzing the image obtained in step (3) for an accumulation of labeled recognition agent characteristic of tissue inflammation. The detection method may also be accomplished in conjunction with the autologous leukocyte activation embodiment of the present invention.

This application features analyzing step (4). Such analysis may be accomplished visually by an experienced diagnostician. That is, diagnosticians familiar with images of inflamed tissue would be able to ascertain that inflammation is or is not present at an imaged tissue site by recognizing patterns characteristic of inflammation in the image obtained from a given patient. Analyzing step (4) may also be accomplished "mechanically" by a computer having a library of previously analyzed images stored in its memory. A match or close correspondence of the test image with a stored image would indicate the presence or absence of inflammation.

A further application of the present invention contemplates a method of monitoring the efficacy of treatment of tissue inflammation in a patient by:

(A) preparing a sequence of diagnostic images of an afflicted tissue site during the treatment, each image being prepared by a process comprising:

(1) labeling a recognition agent, wherein the agent is capable of interacting with activated leukocytes accumulated at the tissue site;

(2) infusing labeled recognition agent into the patient; and

(3) imaging the tissue site; and

(B) analyzing the sequence of images obtained in step (A) to determine response of the patient to the treatment. Again, this monitoring method may be accomplished in conjunction with the autologous leukocyte activation embodiment of the present invention as well.

This aspect of the present invention features serial imaging, i.e., a sequence of diagnostic images of a tissue site prepared over time and accumulated in a storage area, to monitor the efficacy of a treatment protocol. Time lag between images will principally be dictated by the condition being treated and be limited by the time required for the imaging process itself.

Analysis step (B) may be accomplished visually by any experienced diagnostician. That is, diagnosticians familiar with images of inflamed tissue would be able to ascertain that inflammation has increased, decreased or remained approximately the same over time by comparing images of the inflamed tissue taken at various time intervals. In other words, serial images would be compared to determine if the treatment protocol being administered to the patient is successful. A decrease in inflammation, ascertainable from serial images of a single tissue site, would indicate a successful treatment strategy. In fact, a person having less familiarity with inflamed tissue and/or images thereof would be able to make a rough determination of the efficacy of the treatment through a comparison of serial images.

An efficacy analysis may also be accomplished "mechanically" by a computer having the serial images stored in its memory. A data point by data point comparison may then be carried out by the computer to determine whether the inflamed tissue has been successfully treated.

To summarize the examples that follow, Examples I II, III and IV describe the preparation of labeled chemotactic peptide and derivatives thereof; Example V

describes peptide-label conjugation; Examples VI and VII describe methods of imaging tissue sites using labeled chemotactic peptides as recognition agents; Examples VIII and IX describe the preparation of monoclonal antibodies; Examples X and XI describe the preparation of labeled monoclonal antibodies; Examples XII and XIII, XIV and XV describe methods of imaging tissue sites using labeled monoclonal antibodies as recognition agents; Examples XVI and XVII describe diagnostic kits; Examples XVIII and XIX describe methods of monitoring treatment of tissue damage; Example XX describes activation of PMNs; Example XXI describes activation of monocytes; Example XXII describes a method of imaging involving infusion of autologous leukocytes; Example XXIII describes the preparation of a photoaffinity labeled peptide; Example XXIV describes the preparation of activated peptide-photoaffinity label conjugates; and Example XXV describes a method of imaging using such photoaffinity label containing conjugates. These examples are offered as illustrations of the present invention and not as limitations thereof.

EXAMPLE I

Preparation of Labeled Chemotactic Peptide

The chemotactic peptide met-leu-phe having an additional cysteine residue is synthesized using tea-bag methodology and solid phase peptide synthesis procedures described by Merrifield et al. (Biochemistry 21: 5020-31, 1982) and Houghten (Proc. Natl. Acad. Sci. (USA) 82: 5131-35, 1985) or using a commercially available automated synthesizer, such as the Applied Biosystems 430 A or using other standard biochemistry techniques. The peptide is cleaved from the resin using HF and established procedures and extracted with dilute acetic acid. The peptide is lyophilized and is purified using reverse phase HPLC on a Vydac C-4 analytical column (The Separa-

tions Group, Hesperia, CA) and a linear gradient of 0.5-1.0%/min from 100% water + 0.1% v/v trifluoroacetic acid to 100% acetonitrile + 0.1% trifluoroacetic acid. The peptide is N-formylated via reaction with acetic anhydride in 98% formic acid for 1 hour at 25°C as described in J. Am. Chem. Soc. 80: 1154 (1958).

A solution of Tc-99m-tartrate is prepared by adding 1.1 ml of degassed distilled water containing 9% ethanol to 100 μg (ca. 0.43 μmoles) $SnCl_2$, 75 mg (ca. 0.32 mmol) disodium tartrate, and 3.2 mCi sodium (Tc-99m) pertechnetate. This mixture is heated at 50°C with a water bath for 15 min. After cooling in a separate container, 100 μl of the Tc-99m-tartrate solution, 200 μl of 0.2M pH 8.0 sodium phosphate buffer, and 100 μg of prepared chemotactic peptide are admixed. The total volume of the solution is adjusted to 0.5 ml with an aqueous solution of 0.15 M sodium chloride and is incubated at 50°C for 60 min.

EXAMPLE II

Preparation of Stabilized, Labeled Chemotactic Peptide

The chemotactic peptide met-leu-phe having an additional Gly-Gly-Lys moiety is synthesized using tea-bag methodology and solid phase peptide synthesis procedures described by Merrifield et al. (Biochemistry 21: 5020-31, 1982) and Houghten (Proc. Natl. Acad. Sci. (USA) 82: 5131-35, 1985) or using a commercially available automated synthesizer, such as the Applied Biosystems 430 A or using other standard biochemistry techniques. The peptide is cleaved from the resin using HF and established procedures and extracted with dilute acetic acid. The peptide is lyophilized and is purified using reverse phase HPLC on a Vydac C-4 analytical column (The Separations Group, Hesperia, CA), and a linear gradient of 0.5-1.0%/min from 100% water + 0.1% v/v trifluoroacetic acid to 100% acetonitrile + 0.1% trifluoroacetic acid. The

peptide is N-formylated via reaction with acetic anhydride in 98% formic acid for 1 h at 25°C as described in J. Am. Chem. Soc. 80: 1154 (1958). Iodine labeling of the lysine residue is accomplished as described by Panuska and Parker (Analytical Biochemistry 160: 192-201, 1987).

EXAMPLE III

Preparation of Chemotactic Peptide Derivatives

The chemotactic peptide analog boc-L-F-L-F having a chain of amino acids, such as -G-G-G-G-G-Y, at its amino terminus is synthesized as described in Biochim. Biophys. Acta 602: 285 (1980) or using a commercially available automated synthesizer, such as the Applied Biosystems 430 A or using other standard biochemistry techniques. The peptide is lyophilized and is purified using reverse phase HPLC on a Vydac C-4 analytical column (The Separations Group, Hesperia, CA), and a linear gradient cf 0.5-1.0%/minute from 100% water + 0.1% v/v trifluoroacetic acid to 100% acetonitrile + 0.1% trifluoroacetic acid. Iodine labeling of the peptide is accomplished as described in European Patent Application publication No. 0289187. The longer chemotactic peptide derivative enhances target cell label retention.

EXAMPLE IV

Preparation of Chemotactic Peptide Derivatives

The chemotactic peptide analog f-norLeu-L-F-norLeu-Y-K is synthesized as described in Science 205: 1412 (1979) or using a commercially available automated synthesizer, such as the Applied Biosystems 430 A or using other standard biochemistry techniques. The peptide is lyophilized and is purified using reverse phase HPLC on a Vydac C-4 analytical column (The Separations Group, Hesperia, CA), and a linear gradient of 0.5-1.0%/minute from 100% water + 0.1% v/v trifluoroacetic

acid to 100% ac tonitrile + 0.1% trifluoroacetic acid. Radiolab ling of the peptide is accomplished as described in European Patent Application publication no. 0203764. This charged chemotactic peptide derivative enhances target cell label retention.

EXAMPLE V

Peptide-Radiolabel Conjugations

A Tc-99m chelate is conjugated to the unlabeled chemotactic peptide of Examples I, II, III or IV as follows. 75 mCi of Tc-99m chelated by N, N'-bismercaptoacetyl 4,5-diaminopentanoic acid is prepared by dithionite reduction of Tc-99m pertechnetate at basic pH with 25 μg of the N_2S_2 ligand. The acid is activated by adding the above complex at pH 7 in 0.5 ml water to 100 μl of water:acetonitrile (1:9) containing 3.0 mg of 2,3,5,6-tetrafluorophenol and 100 μl of water:acetonitrile (1:9) containing 7.5 mg of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide (morpho CDI). After storing for 18 h at room temperature, the mixture is purified using a Baker-10 SPE reversed phase C_{18} column. The column is conditioned with 2 ml of ethanol and is then washed with HPLC grade water. The reaction mixture is then added to the column, the column is then washed four times with 2 ml volumes of 10% methanol in 0.01 sodium phosphate, pH 7.0 and the ester complex is finally eluted with 2.5 ml portions of acetonitrile.

To a 2 ml vial is added 4.5 mCi of activated ester complex in acetonitrile, the solvent is evaporated in a nitrogen stream, and 0.4 ml of sodium borate (0.5M, pH 9.0) is added. While agitating, the chemotactic peptide is added and incubation at room temperature is conducted for 30 min.

EXAMPLE VI

Method of Imaging -- Chemotactic Peptide as Recognition Agent

50 mCi of the labeled chemotactic peptide of EXAMPLE II is admixed with a pharmaceutically acceptable saline solution. This mixture is administered subcutaneously to the patient. After 24 h, a diagnostic image is prepared through the use of a Raytheon LFOV Anger gamma camera.

EXAMPLE VII

Method of Imaging -- Chemotactic Peptide as Recognition Agent

30 mCi of the labeled chemotactic peptide of EXAMPLE III is admixed with a pharmaceutically acceptable saline solution. This mixture is administered intravenously to the patient. After 18 h, a diagnostic image is prepared through the use of a STARCAM gamma camera made by General Electric Corporation.

EXAMPLE VIII

Monoclonal Antibody Generation

Mice are immunized with homotypic aggregates of activated PMNs pooled from normal donors. This pooling of PMNs is accomplished through the withdrawal of blood (in amounts of approximately 150 cc) by venipuncture in heparinized tubes and mixing with an equal volume of 3% dextran in phosphate-buffered saline (PBS). After sedimentation at 1 x g for 10 min at room temperature, the leukocyte-rich plasma is layered on top of lymphocyte separation medium (LSM, Organon Technica, Durham, NC). PMNs are purified by treating the PMNs and the red blood cell (RBC) pellet with RBC lysing solution (0.8% w/v NH_4Cl, 0.1% w/v $KHCO_3$, 37.0 mg tetrasodium EDTA in 100 ml water at pH 7.3). After incubating approximately 5 min at room temperature, the volume is increased three times with PBS and the cells are washed twice by centrifugation. The PMNs are incubated for 15 to 30 min with

100 U/ml GM-CSF in the presence of human serum to generate activated PMNs which are capable of forming homotypic aggregates.

These aggregated PMNs are then injected into mice as an immunogen. Hybridomas generated by the immunogen are then screened against activated or non-activated pooled PMNs in the presence of human serum. Desirable antibodies are those that recognize activation markers as well as conformation-dependent determinants (in this case, PMN-PMN aggregates). The antibodies identified as desirable are further screened against inflammatory lesions using immunoperoxidase techniques.

EXAMPLE IX

Monoclonal Antibody Generation

Mice are immunized with heterotypic aggregates prepared in vitro and having activated PMNs pooled from normal donors as one component of the heterotypic aggregate. This pooling of PMNs is accomplished through the withdrawal of blood (in amounts of approximately 150 cc) by venipuncture in heparinized tubes and mixing with an equal volume of 3% dextran in phosphate-buffered saline (PBS). After sedimentation at 1 x g for 10 min at room temperature, the leukocyte-rich plasma is layered on top of lymphocyte separation medium (LSM, Organon Technica, Durham, NC). PMNs are purified by treating the PMNs the and red blood cell (RBC) pellet with RBC lysing solution (0.8% w/v NH_4Cl, 0.1% w/v $KHCO_3$, 37.0 mg tetrasodium EDTA in 100 ml water at pH 7.3). After incubating approximately 5 min at room temperature, the volume is increased three times with PBS and the cells are washed twice by centrifugation.

The activated PMNs are incubated with either vascular endothelial cells or portions thereof, bacteria or other pathogenic organisms or target tissue (tissue afflicted with inflammation). This incubation is

conducted for a time sufficient (approximately 30 min) to permit association of the activated PMNs with the vascular endothelial cells, the commencement of phagocytosis with respect to the pathogenic organisms, or attachment of the activated PMNs and the target cells.

One type of heterotypic aggregate is then injected into mice as an immunogen. Hybridomas generated by the immunogen are then screened against activated or non-activated pooled PMNs in the presence of human serum. Desirable antibodies are those that recognize activation markers as well as conformation-dependent determinants (in this case, epitopes present on PMN-vascular endothelium aggregates, PMNs undergoing phagocytosis or PMN-target cell aggregates). The antibodies identified as desirable are further screened against inflammatory lesions using immunoperoxidase techniques.

EXAMPLE X

Antibody-Radiolabel Conjugation

In an evacuated vial is combined 100 μl of water, 100 μl acetonitrile, 100 μl of citrate solution (28.8 mg; 1.5 x 10^{-4} mol), 50 μl of ligand (tetrafluorophenyl 4,5-di-(tetrahydropyranylmercaptoacetamido)pentanoate; 0.40 mg; 6.5 x 10^{-7} mol), 50 μl of stannous chloride (0.5 mg; 2.6 x 10^{-6} mol), and 50 μl of Tc-99m in acetonitrile (4.25 mg; 2.3 x 10^{-8} mol). The mixture is heated at 50°C for 1 h and then 0.30 ml of 1N NaOH is added.

The tetrafluorophenyl ester product of the Tc-99m N_2S_2 complex is purified on a C_{18} Baker-10 SPE column. After application to the column, impurities are washed off with 2 x 3 ml of water and 4 x 3 ml of 10% CH_3OH/ 0.01M phosphate, pH 7. The product is eluted with 2 ml of acetonitrile and then the solution is reduced to dryness under a stream of nitrogen.

Conjugation of the Tc-99m N_2S_2 complex is done by addition of the antibody of Example VIII or Example IX

to the complex in borate buffer (0.5M, pH 9). Incubation is maintained for 30 min at room temperature.

EXAMPLE XI

Antibody-Radiolabel Conjugations

A Tc-99m chelate is conjugated to the monoclonal antibody of Example VIII or Example IX as follows. 75 mCi of Tc-99m chelated by N, N'-bismercaptoacetyl 4,5-diaminopentanoic acid is prepared by dithionite reduction of Tc-99m pertechnetate at basic pH with 25 μg of the N_2S_2 ligand. The acid is activated by adding the above complex at pH 7 in 0.5 ml water to 100 μl of water: acetonitrile (1:9) containing 3.0 mg of 2,3,5,6-tetrafluorophenol and 100 μl of water:acetonitrile (1:9) containing 7.5 mg of 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide (morpho CDI). After storing for 18 h at room temperature, the mixture is purified using a Baker-10 SPE reversed phase C_{18} column. The column is conditioned with 2 ml of ethanol and is then washed with HPLC grade water. The reaction mixture is then added to the column, the column is then washed four times with 2 ml volumes of 10% methanol in 0.01 sodium phosphate, pH 7.0 and the ester complex is finally eluted with 2.5 ml portions of acetonitrile.

To a 2 ml vial is added 4.5 mCi of activated ester complex in acetonitrile, the solvent is evaporated in a nitrogen stream, and 0.4 ml of sodium borate (0.5 M, pH 9.0) is added. While agitating, the antibody is added and incubation at room temperature is conducted for 30 min.

EXAMPLE XII

Method of Imaging -- Monoclonal Antibody as Recognition Agent

30 mCi of the lab led monoclonal antibody of EXAMPLE XI is admixed with a pharmaceutically acceptable saline solution. This mixture is administered intraperitoneally

to the patient. After 18 h, a diagnostic image is prepared through the use of a STARCAM gamma camera made by General Electric Corporation.

EXAMPLE XIII

Method of Imaging -- Dual (Cold and Hot) Monoclonal Antibody Administration

10 mg of non-labeled monoclonal antibody of EXAMPLE VIII is admixed with a pharmaceutically acceptable saline solution. This mixture is administered intravenously to the patient 30 min prior to radiolabeled antibody. 30 mCi of the labeled monoclonal antibody of EXAMPLE X is admixed with a pharmaceutically acceptable saline solution and administered to the patient intravenously. Following the passage of 3-8 additional h, a diagnostic image is prepared through the use of a STARCAM gamma camera made by General Electric Corporation.

EXAMPLE XIV

Method of Imaging -- Monoclonal Antibody as Recognition Agent

20 mCi of the labeled monoclonal antibody of EXAMPLE XI is admixed with a pharmaceutically acceptable saline solution. This mixture is administered intravenously to the patient. After 24 h, a diagnostic image is prepared through the use of a Raytheon LFOV Anger gamma camera. A diagnostician examines the prepared image and determines whether the image is characteristic of tissue having damage mediated by inflammation.

EXAMPLE XV

Method of Imaging -- Dual (Cold and Hot) Monoclonal Antibody Administration

7.5 milligrams of non-labeled monoclonal antibody of EXAMPLE IX is admixed with a pharmaceutically acceptable saline solution. This mixture is administered intravenously to the patient. 5 min later, 0.5 mg of the

labeled monoclonal antibody of EXAMPLE XI is admixed with a pharmaceutically acceptable saline solution and administered to the patient subcutaneously. Following the passage of 12 additional h, a diagnostic image is prepared through the use of Raytheon LFOV Anger gamma camera. A diagnostician examines the prepared image and determines whether the image is characteristic of tissue having damage resulting from a transient decrease in blood flow to that tissue.

EXAMPLE XVI

Diagnostic Kit

Lyophilized recognition agent of EXAMPLE VIII is contained in a sterile vial. A buffer of pharmaceutically acceptable solution is contained in an another sterile vial. Both vials are contained in a box. Instructions regarding the labeling and use of the recognition agent are printed on a label affixed to the box by an adhesive.

EXAMPLE XVII

Diagnostic Kit--Dual Administration Method

Lyophilized recognition agent of EXAMPLE VIII is contained in two sterile vials. Pharmaceutically acceptable solution is contained in a third sterile vial. All three vials are housed in a box in which the instructions for the use of the kit are contained in a separate pamphlet.

EXAMPLE XVIII

Monitoring Method

During steroid treatment of inflammation, diagnostic images are prepared as in EXAMPLE XIV at 72 h intervals. Th series of images is examined to determine the fate of the damaged tissue over time. An observed decrease in

the amount of labeled recognition agent in the area of tissue damage over time indicates treatment success.

EXAMPLE XIX

Monitoring Method

During the course of treatment of cardiac ischemia, diagnostic images are prepared as in Example XV at 72 h intervals. The series of images is examined to determine the fate of the damaged tissue over time. An observed increase in the amount of labeled recognition agent in the area of tissue damage over time indicates treatment failure and a danger of myocardial infarction.

EXAMPLE XX

Activation of Polymorphonuclear Leukocytes

Cytophoresis is performed on a patient, in order to obtain from peripheral blood a fraction enriched for mature PMNs. Briefly, the PMN enrichment technique involves standard blood phoresis performed in combination with hydroxyethyl starch, a sedimenting agent. The patient may also be pretreated with prednisone for 12 to 18 h immediately preceding the phoresis process. Prednisone is a steroid that induces release of mature neutrophils from the bone marrow to the peripheral blood. The PMNs are collected under sterile conditions, with a typical cellular recovery approximating 30 x 10^9 cells/ cytophoresis process.

The harvested PMNs are incubated for 15 to 30 min with 100 U/ml GM-CSF (recombinant human GM-CSF may be obtained from a COS cell transfectant (D. Metcalf et al., Blood 67:37-45, 1986)) in order to generate activated PMNs.

EXAMPLE XXI

Activation of Macrophages

Peripheral blood from patients is obtained via venipuncture and fractionated by density centrifugation. That is, heparinized blood is layered onto Ficoll-Paque (Pharmacia), the gradient is centrifuged and the mononuclear cells are harvested from the plasma-gradient interface. The harvested cells are washed twice in serum-free RPMI 1640 medium. Monocytes are collected by adhering interface cells in RPMI 1640 containing 10% fetal calf serum and penicillin/streptomycin at $5x10^6$ cells/ml. Adherent monocytes are incubated in the presence of a low pyrogen content M-CSF preparation (20 ng/ml, Genetics Institute) for 72 h.

EXAMPLE XXII

Activated Autologous PMN/Labeled I-CAM Interaction Inhibitor Conjugate

Fab fragments of 4F-2 [an anti-human monocyte antibody produced by the hybridoma cell line designated "4F2C13", ATCC, Rockville, MD] capable of blocking LFA/I-CAM interaction are radiolabeled in accordance with the procedure described in European Patent Application Publication No. 0188256. Activated PMNs prepared in accordance with Example XX are incubated with these radiolabeled antibody fragments for 60 min. Conjugates formed from this incubation are then infused into a patient.

Additional radiolabeled antibody fragments are infused into a patient together with the conjugates prepared above. A diagnostic image is then prepared through the use of a STARCAM gamma camera made by General Electric Corporation.

EXAMPLE XXIII

Preparation of a Photoaffinity Labeled Peptide

The photoaffinity labeled chemotactic peptide met-leu-phe-gly-gly-(p-benzoyl-L-phe) is synthesized, cleaved from the resin and purified as described in J. Biol. Chem. 261:10695 (1986). The resulting peptide is then N-formylated via reaction with acetic anhydride in 98% formic acid for 1 h at 25°C as described in J. Am. Chem. Soc. 80: 1154 (1958). Iodine labeling of the lysine residue is accomplished as described by Panuska and Parker (Analytical Biochemistry 160: 192-201, 1987).

EXAMPLE XXIV

Activated Peptide-Photoaffinity Label Conjugates

Cytophoresis is performed on a patient, in order to obtain from peripheral blood a fraction enriched for mature PMNs. Briefly, the PMN enrichment technique involves standard blood phoresis performed in combination with hydroxyethyl starch, a sedimenting agent. The patient may also be pretreated with prednisone for 12 to 18 h immediately preceding the phoresis process. Prednisone is a steroid that induces release of mature neutrophils from the bone marrow to the peripheral blood. The PMNs are collected under sterile conditions, with a typical cellular recovery approximating 30 x 10^9 cells/ cytophoresis process.

50 μM of the photoaffinity and radionuclide labeled peptide of Example XXIII (i.e., a concentration of peptide just sufficient to saturate the leukocyte chemotactic peptide receptors) is incubated with the obtained leukocytes for about 15 to 30 min to permit binding of such leukocytes and peptide. The resultant mixture is photolyzed via an array of lamps as described in J. Biol. Chem. 261:10695 (1986), whereupon a triplet biradical affinity reagent is generated. This reagent now covalently binds to the leukocyte surface.

EXAMPLE XXV

Method of Imaging -- Photoaffinity Labeled Peptide as Recognition Agent

50 mCi of the stabilized, radionuclide labeled chemotactic peptide of EXAMPLE XXIV is admixed with a pharmaceutically acceptable saline solution. This mixture is administered subcutaneously to the patient. After 18 h, a diagnostic image is prepared through the use of a Raytheon LFOV Anger gamma camera.

WHAT IS CLAIMED IS:

1. A method of imaging a tissue site of inflammation comprising:

(1) infusing non-labeled recognition agent into a patient, wherein said agent is capable of interacting with activated leukocytes accumulated at said tissue site;

(2) infusing labeled recognition agent into the patient; and

(3) imaging said tissue site,

whereby medical conditions involving tissue inflammation may be detected, evaluated and monitored.

2. A method of claim 1 wherein said labeled recognition agent includes a chelating moiety formed from a compound selected from the group consisting of N_2S_2, N_3S, N_2S_3, N_2S_4, and N_3S_3.

3. A method of claim 1 wherein said tissue site of inflammation is an hypoxic tissue site in a patient suffering from a condition characterized by transient decrease in blood flow to said tissue site.

4. A method of claim 3 wherein said tissue site is ischemic heart muscle or bowel tissue.

5. A method of claim 3 wherein said tissue site is afflicted with collagen vascular dysfunction or with cancer.

6. A method of claim 1 wherein said leukocytes are polymorphonuclear leukocytes or monocytes.

7. A method of claim 1 wherein said labeled recognition agent is labeled with ^{111}In or ^{99m}Tc.

8. A method of claim 1 wherein said labeled recognition agent is a labeled monoclonal antibody or fragment thereof directed against a leukocyte activation marker.

9. A method of claim 8 wherein said activation marker is an epitope of a leukocyte-associated antigen which exhibits enhanced cell surface expression following activation.

10. A method of claim 9 wherein said activation marker is an epitope of a leukocyte surface antigen involved in chemotaxis, phagocytosis or cell killing.

11. A method of imaging tissue sites of inflammation comprising:

(1) infusing a labeled recognition agent capable of interacting selectively with activated leukocytes accumulated at said tissue sites into a patient; and

(2) imaging said tissue sites,

whereby medical conditions involving tissue damage mediated by inflammation may be detected, evaluated and monitored.

12. A method of claim 11 wherein said labeled recognition agent includes a chelating moiety formed from a compound selected from the group consisting of N_2S_2, N_3S, N_2S_3, N_2S_4, and N_3S_3.

13. A method of claim 11 wherein said tissue sites of inflammation are hypoxic tissue sites in a patient suffering from a condition characterized by transient decrease in blood flow to said tissue sites.

14. A method of claim 13 wherein said tissue sites are ischemic heart muscle or bowel tissue.

15. A method of claim 13 wherein said tissue sites are afflicted with collagen vascular dysfunction or with cancer.

16. A method of claim 11 wherein said leukocytes are polymorphonuclear leukocytes or monocytes.

17. A method of claim 11 wherein said labeled recognition agent is labeled with ^{111}In or ^{99m}Tc.

18. A method of claim 11 wherein said labeled recognition agent is a labeled monoclonal antibody or fragment thereof directed against a leukocyte activation marker.

19. A method of claim 18 wherein said activation marker is an epitope of a leukocyte-associated antigen which exhibits enhanced cell surface expression following activation.

20. A method of claim 19 wherein said activation marker is an epitope of a leukocyte surface antigen involved in chemotaxis, phagocytosis or cell killing.

21. A method of claim 11 wherein said labeled recognition agent is capable of recognizing a conformation-dependent determinant exposed or formed upon homotypic aggregation of leukocytes or heterotypic aggregation of leukocytes and damaged tissue.

22. A method of claim 21 wherein said labeled recognition agent is a labeled monoclonal antibody or fragment thereof directed against a heterotypic aggregate

of activated polymorphonuclear leukocytes and damaged tissue.

23. A method of claim 21 wherein said labeled recognition agent is a labeled monoclonal antibody or fragment thereof directed against a homotypic aggregate of activated polymorphonuclear leukocytes.

24. A method of claim 11 wherein said labeled recognition agent is capable of recognizing a complement component or a fragment, a derivative or an analog thereof or a complement receptor.

25. A method of claim 24 wherein said complement receptor is a receptor for C3, Clq, C3a or C5a.

26. A method of claim 24 wherein said labeled recognition agent is a labeled monoclonal antibody or fragment thereof directed against a complement component, or a fragment, a derivative or an analog thereof bound at said tissue sites.

27. A method of claim 26 wherein said labeled monoclonal antibody or fragment thereof is directed against C3dg.

28. A method of claim 26 wherein said complement component is C3a, C5a or C3a des Arg.

29. A method of claim 11 wherein said labeled recognition agent is capable of recognizing a chemotactic peptide, or a fragment, a derivative or an analog thereof or a chemotactic peptide receptor.

30. A method of claim 29 wherein said chemotactic peptide receptor is a receptor for f-met-leu-phe.

31. A method of claim 29 wherein said labeled recognition agent is a labeled chemotactic peptide.

32. A method of claim 31 wherein said labeled chemotactic peptide is labeled f-met-leu-phe.

33. A method of claim 31 wherein said labeled chemotactic peptide is radiolabeled via an additional tyrosine, lysine, cysteine or phenylalanine residue or an analog thereof synthesized as part of the peptide.

34. A method of claim 31 wherein D-amino acids are incorporated into said labeled chemotactic peptide during synthesis, thereby decreasing in vivo degradation of said peptide.

35. A method of claim 31 further comprising:

(3) comparing inflammation site localization of said labeled chemotactic peptide and reticuloendothelial system localization of said labeled peptide, wherein exhibition of a substantial affinity of said labeled peptide for circulating or reticuloendothelial cells necessitates step (4); and, if necessary,

(4) altering the amino acid sequence of the labeled peptide through addition or deletion of amino acids, so as to more closely correlate peptide structure with that bound by high affinity receptors of the activated leukocytes and less closely correlate with that bound by receptors of non-activated leukocytes,

thereby producing a modified labeled peptide capable of preferentially binding to activated leukocytes at sites of inflammation.

36. A method of claim 11 wherein said labeled recognition agent is capable of recognizing LFA-1, LFA-2,

LFA-3, CD2, an immunoglobulin receptor or a leukotriene receptor.

37. A method of claim 11 wherein said labeled recognition agent is labeled leukotriene.

38. A method of claim 11 wherein said labeled recognition agent is a labeled eosinophilotactic peptide.

39. A method of imaging a tissue site of inflammation comprising:

(1) withdrawing leukocytes from a patient;

(2) incubating leukocytes of step (1) with a labeled recognition agent capable of interacting with a leukocyte binding moiety;

(3) infusing into said patient labeled recognition agent and leukocytes incubated in step (2); and

(4) imaging said tissue site,

whereby medical conditions involving tissue inflammation may be detected, evaluated and monitored.

40. A method of claim 39 further comprising the step of activating leukocytes of step (1).

41. A method of claim 40 wherein said activating step is accomplished by incubating withdrawn leukocytes of step (1) with an activating agent.

42. A method of claim 41 wherein said leukocytes are polymorphonuclear leukocytes or monocytes.

43. A method of claim 41 wherein said activating agent is an agent capable of generating an oxidative burst in leukocytes.

44. A method of claim 41 wherein said activating agent is GM-CSF, G-CSF, gamma interferon, a calcium ionophore, M-CSF, CSF-1, gamma interferon or TNF.

45. A method of claim 39 wherein said labeled recognition agent includes a chelating moiety formed from a compound selected from the group consisting of N_2S_2, N_3S, N_2S_3, N_2S_4, and N_3S_3.

46. A method of claim 39 wherein said tissue site of inflammation is an hypoxic tissue site in a patient suffering from a condition characterized by transient decrease in blood flow to said tissue site.

47. A method of claim 39 which further comprises infusing non-labeled recognition agent prior to infusing step (3).

48. A method of claim 46 wherein said tissue site is ischemic heart muscle or bowel tissue.

49. A method of claim 46 wherein said tissue site is afflicted with collagen vascular dysfunction or with cancer.

50. A method of claim 39 wherein said labeled recognition agent is labeled with ^{111}In or ^{99m}Tc.

51. A method of claim 39 wherein said leukocyte binding moiety is an adhesion protein receptor.

52. A method of claim 51 wherein said leukocyte binding moiety is LEU-CAM.

53. A method of claim 39 wherein said leukocyte binding moiety is an adhesion protein.

54. A method of claim 53 wherein said adhesion protein is LFA-1, LFA-2 or LFA-3.

55. A method of claim 51 wherein said labeled recognition agent is an agent capable of inhibiting LFA interaction with I-CAM, LEU-CAM or a substantially functional equivalent thereof.

56. A method of claim 55 wherein said labeled recognition agent is a labeled monoclonal antibody or a fragment thereof.

57. A method of claim 55 wherein said labeled recognition agent is labeled I-CAM or a substantially functional equivalent sequence.

58. A method of claim 39 further comprising infusing an anti-LFA Fab or $F(ab')_2$ fragment into the patient concurrently or in close temporal relation with infusing step (3).

59. A method of claim 39 wherein said leukocyte binding moiety is a chemotactic peptide receptor.

60. A method of claim 59 wherein said chemotactic peptide receptor is a receptor for f-met-leu-phe.

61. A method of claim 59 wherein said labeled recognition agent is a labeled chemotactic peptide, or a fragment, a derivative or an analog thereof or a labeled chemotactic peptide receptor inhibitor.

62. A method of claim 61 wherein said labeled chemotactic peptide is labeled f-met-leu-phe.

63. A method of claim 61 wherein said labeled chemotactic peptide is radiolabeled via an additional tyrosine, lysine, cysteine or phenylalanine residue or analog thereof synthesized as part of the peptide.

64. A method of claim 61 wherein D-amino acids are incorporated into said labeled chemotactic peptide during synthesis, thereby decreasing in vivo degradation of said peptide.

65. A method of claim 61 further comprising:

(5) comparing inflammation site localization of said labeled chemotactic peptide and reticuloendothelial system localization of said labeled peptide, wherein exhibition of a substantial affinity of said peptide for circulating or reticuloendothelial cells necessitates step (6); and, if necessary,

(6) altering the amino acid sequence of the labeled peptide through addition or deletion of amino acids, so as to more closely correlate peptide structure with that bound by high affinity receptors of the activated leukocytes and less closely correlate with that bound by receptors of non-activated leukocytes,

thereby producing a modified labeled peptide capable of preferentially binding to activated leukocytes at sites of inflammation.

66. A method of claim 39 wherein said leukocyte binding moiety is a complement receptor.

67. A method of claim 66 wherein said complement receptor is the C3a or C5a receptor.

68. A method of claim 39 wherein said leukocyte binding moiety is a leukocyte surface antigen which up-regulates upon leukocyte activation.

69. A method of claim 68 wherein said labeled recognition agent is a labeled Fab or $F(ab')_2$ fragment of a monoclonal antibody capable of recognizing an up-regulated leukocyte surface antigen.

70. A monoclonal antibody directed against a heterotypic aggregate of activated leukocytes.

71. A monoclonal antibody directed against a homotypic aggregate of activated leukocytes.

72. A method of imaging a tissue site of inflammation comprising:

(1) withdrawing leukocytes from a patient;

(2) constructing a chemotactic peptide or a fragment, a derivative or analog thereof containing an affinity label and a radionuclide label;

(3) incubating leukocytes of step (1) with the peptide of step (2) for a time sufficient to permit binding thereof;

(4) activating said affinity label;

(5) infusing into the patient labeled peptide and leukocytes resulting from step (4); and

(6) imaging said tissue site,

whereby medical conditions involving tissue inflammation may be detected, evaluated and monitored.

73. A method of claim 72 wherein said affinity label is a photoaffinity label.

74. A method of claim 72 wherein said peptide is of the formula:

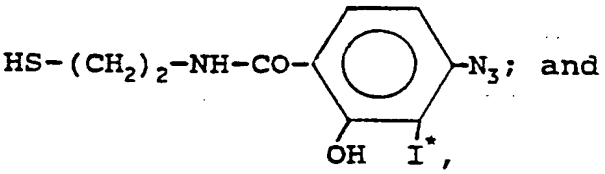
f-M-L-F-spacer-X,

wherein X is selected from the group consisting of tyrosine, cysteine or lysine.

75. A method of claim 74 wherein said spacer is 0 to about 5 glycine moieties.

76. A method of claim 73 wherein said photoaffinity label is p-benzoyl-L-phe.

77. A method of claim 73 wherein said photoaffinity label is



wherein I^* is radioactive iodine.

78. A method of claim 73 wherein said photoaffinity label is N-(4-(4'-azido-3'-[^{125}I]iodophenylazo)benzoyl)-N'-hydroxysuccinimide.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/01399

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL.[5] A61K 49/02
U.S. CL. 424/1.1

II. FIELDS SEARCHED

Classification System	Minimum Documentation Searched [7] Classification Symbols
U.S.	424/1.1,85.91 514/2 530/388,402

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	US, A, 4,634,586 GOODWIN ET AL. 06 JANUARY 1987 (See Columns 1-4)	11,13-15,17-19 1-10,12,16, 20-24,70,71
X Y	US, A, 4,443,426 THAKUR 17 APRIL 1984 (See Columns 2-4)	11,12,17-19 1-10,13-16, 20-24,70,71
Y	WO, A, WO88/02594 NEORX 21 APRIL 1988 (See pages 3-9)	1-10, 12, 16 20-24,70,71
Y	US, A, 4,153,417 HALLGREN ET AL 08 MAY 1979 (See Columns 1-2)	25,26
Y	US, A, 4,314,987 MORRIS ET AL. 09 FEBRUARY 1982 (See Columns 2-4)	25,26,28
T	US, A, 4,925,648 HANSEN ET AL. 15 MAY 1990	

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
15 JUNE 1990	25 JUL 1990
International Searching Authority	**Signature of Authorized Officer**
ISA/US	John S. Maples

Form PCT/ISA/210 (second sheet) (Rev.11-87)

FURTHER INFORMATI N CON..NUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

I. Claims 1-38, 70 and 71 drawn to a first method of imaging a tissue site classified in Class 424 subclass 1.1.

II. Claims 39-69 drawn to a second method of imaging a tissue site classified in Class 424 subclass 1.1.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

1-38, 70,71

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INF RMATI N CONTINUED FROM THE ~~FIRST SHEET~~ OBSERVATIONS-UNITY OF INVENTION
(Not for publication)

III. Claims 72-78 drawn to a third method of imaging a tissue site classified in Class 424 subclass 1.1.

FURTHER INFORMATION CONTINUED FROM PCT TELEPHONE MEMORANDUM

I. Claims 1-38, 70 and 71 drawn to a first method of imaging a tissue site classified in Class 424 subclass 1.1.

II. Claims 39-69 drawn to a second method of imaging a tissue site classified in Class 424 subclass 1.1.

III. Claims 72-78 drawn to a third method of imaging a tissue site classified in Class 424 subclass 1.1.

The above inventions lack unity under PCT Rule 13, since, for example, the Group I method does not include the particular step of the Group II and III inventions of withdrawing leukocytes from a patient. Also, the Group III invention includes an additional affinity label which the Group II invention does not comprise.

INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen
PCT/DE 96/01824

Feld I Bemerkungen zu den Ansprüchen, die sich als nicht recherchierbar erwiesen haben (Fortsetzung von Punkt 1 auf Blatt 1)

Gemäß Artikel 17(2)a) wurde aus folgenden Gründen für bestimmte Ansprüche kein Recherchenbericht erstellt:

1. [X] Ansprüche Nr.
weil Sie sich auf Gegenstände beziehen, zu deren Recherche die Behörde nicht verpflichtet ist, nämlich

Bemerkung: Obwohl der(die) Anspruch(üche) 16 sich auf ein Verfahren zur Behandlung des menschlichen/tierischen Kwrpers bezieht(en), wurde die Recherche durchgeführt und gründete sich auf die angeführten Wirkungen der Verbindung/Zusammensetzung.

2. [X] Ansprüche Nr. 1-16
weil sie sich auf Teile der internationalen Anmeldung beziehen, die den vorgeschriebenen Anforderungen so wenig entsprechen, daß eine sinnvolle internationale Recherche nicht durchgeführt werden kann, nämlich

Wegen der großen Zahl der durch den Anspruchwortlaut definierten Verbindungen wurde die Recherche für den Grundgedanken der Anmeldung und die in der Beschreibung erwähnten Beispiele durchgeführt.

3. [ ] Ansprüche Nr.
weil es sich dabei um abhängige Ansprüche handelt, die nicht entsprechend Satz 2 und 3 der Regel 6.4 a) abgefaßt sind.

Feld II Bemerkungen bei mangelnder Einheitlichkeit der Erfindung (Fortsetzung von Punkt 2 auf Blatt 1)

Die internationale Recherchenbehörde hat festgestellt, daß diese internationale Anmeldung mehrere Erfindungen enthält:

1. [ ] Da der Anmelder alle erforderlichen zusätzlichen Recherchengebühren rechtzeitig entrichtet hat, erstreckt sich dieser internationale Recherchenbericht auf alle recherchierbaren Ansprüche der internationalen Anmeldung.

2. [ ] Da für alle recherchierbaren Ansprüche die Recherche ohne einen Arbeitsaufwand durchgeführt werden konnte, der eine zusätzliche Recherchengebühr gerechtfertigt hätte, hat die Internationale Recherchenbehörde nicht zur Zahlung einer solchen Gebühr aufgefordert.

3. [ ] Da der Anmelder nur einige der erforderlichen zusätzlichen Recherchengebühren rechtzeitig entrichtet hat, erstreckt sich dieser internationale Recherchenbericht nur auf die Ansprüche der internationalen Anmeldung, für die Gebühren entrichtet worden sind, nämlich auf die Ansprüche Nr.

4. [ ] Der Anmelder hat die erforderlichen zusätzlichen Recherchengebühren nicht rechtzeitig entrichtet. Der internationale Recherchenbericht beschränkt sich daher auf die in den Ansprüchen zuerst erwähnte Erfindung; diese ist in folgenden Ansprüchen erfaßt:

Bemerkungen hinsichtlich eines Widerspruchs

[ ] Die zusätzlichen Gebühren wurden vom Anmelder unter Widerspruch gezahlt.

[ ] Die Zahlung zusätzlicher Gebühren erfolgte ohne Widerspruch.

INTERNATIONALER RECHERCHENBERICHT

Angaben zu Veröffentlichungen, die zur selben Patentfamilie gehören

Internationales Aktenzeichen
PCT/DE 96/01824

Im Recherchenbericht angeführtes Patentdokument	Datum der Veröffentlichung	Mitglied(er) der Patentfamilie	Datum der Veröffentlichung
DE 4301871 A	14-07-94	AU 666059 B	25-01-96
		AU 5314694 A	21-07-94
		CA 2113245 A	14-07-94
		EP 0606683 A	20-07-94
		JP 7149799 A	13-06-95
		ZA 9400186 A	18-08-94
WO 9315771 A	19-08-93	US 5310536 A	10-05-94
		AU 3610593 A	03-09-93
		EP 0630264 A	28-12-94
		JP 7503732 T	20-04-95
EP 0403243 A	19-12-90	CA 2019035 A	16-12-90
		DE 69023394 D	14-12-95
		DE 69023394 T	04-07-96
		JP 2512604 B	03-07-96
		JP 3081295 A	05-04-91
		US 5248764 A	28-09-93
WO 9409128 A	28-04-94	AU 5537994 A	09-05-94
		US 5571713 A	05-11-96
WO 9519791 A	27-07-95	CA 2180555 A	27-07-95
		EP 0743861 A	27-11-96
WO 9506633 A	09-03-95	AU 7528894 A	22-03-95
		CA 2169269 A	09-03-95
		EP 0716647 A	19-06-96
		US 5574140 A	12-11-96
WO 9400489 A	06-01-94	AU 4768893 A	24-01-94
		CA 2138647 A	06-01-94
		EP 0649434 A	26-04-95
		JP 8503924 T	30-04-96
		US 5620675 A	15-04-97
WO 9532192 A	30-11-95	CA 2190727 A	30-11-95
		EP 0759913 A	05-03-97